IDENTIFICATION OF MECHANISMS OF BENEFICIAL EFFECTS OF DIETARY CLAYS IN PIGS AND CHICKS DURING AN ENTERIC INFECTION

BY

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DISSERTATION

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ABSTRACT: Dietary clays can reduce diarrhea in pigs, but the mechanisms are unknown. Of several candidates, strengthening of the intestinal barrier is most solidly supported by the literature. Three approaches were taken in order to elucidate some of those mechanisms. First, pigs were challenged with a pathogenic *E.coli* in order to determine how the challenge and 3 different dietary clays affect the intestinal barrier of pigs. The challenge reduced the barrier integrity as indicated by increased bacterial translocation from the intestinal lumen to mesenteric lymph nodes (from 0.76 $\log_{10}$CFU/g of lymph node for the sham group to 1.93 $\log_{10}$CFU/g of lymph node on average), increased crypt depth in the ileum (from 210.9 um in the sham group to 227.3 um in the challenged group on average), and increased goblet cell size (from 30.36 μm^2 in the sham group to 32.38 μm^2 in the challenged group on average) and number (from 24.60 in the sham group to 27.93 in the challenged group) in the ileum , thus reducing growth performance. One of the clays, smectite A (**SMA**) increased goblet cell size (from 29.58 μm^2 in the sham group to 35.96 μm^2 in the challenged group) during the acute phase of the infection, indicating increased mucus production. Second, chicks were challenged with *Salmonella enterica* serovar Typhimurium with the objective to test the beneficial effects of 3 different dietary clays on growth performance and barrier function during the chronic phase of infection. Challenge with *Salmonella enterica* serovar Typhimurium reduced performance by 11%; during d 3-7 post infection the ADG was reduced from 54.83 g in the sham group to 48.82 g in the challenged group. The challenge increased goblet cell size (from 23.3 μm^2 in the sham group to 29.7 μm^2 in the challenged group fed basal diet) and number (from 99.50 in the sham group to 131.98 in the challenged group fed the basal diet), and reduced villus height. The clays restored performance in the challenged group. One of the clays (SMA) reduced goblet cell size (from 29.7 μm^2 in the challenge group fed basal diet to 23.4 μm^2 in the challenged group fed SMA) and number (from

131.98 in the challenge group fed basal diet to 99.78 in the challenged group fed SMA), indicating increased secretion of mucus in the chronic phase of infection, thus strengthening the barrier. The apparent difference in results between the 2 experiments can be explained by the fact that measurements were done during different phases of infection in the pigs and chicks. When pigs were challenged with *E.coli* the measurements were done during the acute phase of infection and when the chicks were challenged with *Salmonella enterica* serovar Typhimurium the measurements were done during the chronic phase of infection. Different clays appeared to work through different mechanisms. All 3 clays restored performance (ADG and ADFI) in the chick experiment but not all 3 clays seemed to enhance the intestinal barrier. Third, a human colorectal adenocarcinoma cell line (LS174T) was grown in the presence or absence of different concentrations of SMA in order to explore potential mechanisms through which SMA may produce the beneficial effects previously observed *in vivo*. The mucin 2 (***MUC2***) expression was down-regulated by SMA and the reason is unclear. The resistin like molecule beta (***RELMβ***) expression was up-regulated by SMA. The magnitude of the up-regulation of *RELMβ* (41%) was greater than the magnitude of the down-regulation of *MUC2* (25%) when LS174T cells were grown in the presence of 0.10% SMA (combined results of 3 cell cultures), thus the further focus was on RELMβ. The secretion of RELMβ by LS174T cells was increased by SMA, with depletion of goblet cells. These observations are in agreement with the results from the enteric challenge in chicks and indicate strenghtening of the mucus barrier.

Key words: dietary clays, goblet cells, smectite

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LIST OF ABBREVIATIONS

AA	Amino acid
AB	Alcian blue
ADFI	Average daily feed intake
ADG	Average daily gain
α-1-AGP	Alpha-1-acid glycoprotein
Al	Aluminum
Ala	Alanine
APPs	Acute phase protein
Arg	Arginine
Asp	Aspartate
ATCC	American Type Culture Collection
BW	Body weight
°C	Degrees Celsius
Ca	Calcium
CA	California
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
Cd	Cadmium
CD	Cluster of differentiation
CFU	Colony forming unit
Cl	Chloride
CO_2	Carbon dioxide

CON	Control
CP	Crude protein
Ct	Cycle threshold
Cu	Copper
Cys	Cysteine
D	Diet
d	Days
DM	Dry matter
E	Escherichia coli
E. coli	Escherichia coli
EDTA	Ethylene diamine tetra acetic acid
e.g.	That is
ELISA	enzyme-linked immunosorbent assay
et al.	And others
ETEC	Enterotoxigenic *Escherichia coli*
FDA	Food and drug administration
Fe	Iron
g	Grams
g	Gravity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G:F	Gain to feed ratio
GUSβ	ß-glucuronidase
Glu	Glutamate

Gly	Glycine
h	Hour
H	Hydrogen
HBSS	Hank's buffered salt solution
HCl	Hydrogen chloride
HCO_3	Bicarbonate
HID	High iron diamine
His	Histidine
H_2O	Water
HSCAS	Hydrate sodium calcium aluminosilicate
I	Iodine
IA	Iowa
i.e.	That is
IFN	Interferon
IL	Illinois
IL	Interleukin
Ile	Isoleucine
Int.	International
K	Potassium
kcal	Kilocalories
kDa	Kilodaltons
kg	Kilograms
KLF4	Krüppel like factor 4

KO	Knockout
Leu	Leucine
LPS	Lipopolysaccharide
LS174T	Human colorectal adenocarcinoma cell line
LT	Heat labile
Lys	Lysine
m	meter
ME	Metabolizable energy
MEM	Minimum essential medium
Met	Methionine
Mg	Magnesium
mg	Milligrams
min	Minutes
MMT	Montmorillonite
Mn	Manganese
mRNA	Messenger ribonucleic acid
MUC	Mucin
μg	Microgram
μm	Micrometer
N	Nitrogen
n	sample size
Na	Sodium
NC	North Carolina

NDP	NanoZoomer Digital Pathology System
NH_4	Ammonium
NJ	New Jersey
NK	Natural Killer
nm	Nanometer
NRC	National Research Council
P	Phosphorus
PA	Pennsylvania
PAMPs	Pathogen associated molecular patterns
PAS	Periodic acid schiff
Pb	Plumb
PBS	Phosphate buffered saline
Phe	Phenylalanine
Pro	Proline
PRRs	Pattern recognition receptors
PVDF	polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RELM	Resistin like molecule
S	*Salmonella*
SALM	*Salmonella*
SAS	Statistical Analysis System
SBM	Soybean meal
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Se	Selenium
Ser	Serine
Si	Silicon
SI	Small intestine
SM	Smectite
Sr	Strontium
ST	Heat stable
ST	*Salmonella enterica* serovar Typhimurium
S. typhimurium	*Salmonella enterica* serovar Typhimurium
TFF	Trefoil factor
Thr	Threonine
Trp	Tryptophan
TX	Texas
Tyr	Tyrosine
U.S.	United States
USDA	United States Department of Agriculture
UT	Utah
Val	Valine
vs.	Versus
wk	Week
χ^2	Chi square
ZEO	Zeolite
Zn	Zinc

CHAPTER 1

INTRODUCTION

Intestinal mucosa is always exposed to a variety of antigens that can disrupt homeostasis. Consequently, pigs and poultry are susceptible to intestinal diseases, thus reducing their productive performance. In order to cope with these antigens, the animals can rely on innate and adaptive immunity and we will focus on the intestinal barrier (which is part of the innate immunity). However, there are some pathogens such as *E. coli* and *Salmonella* that have specialized systems that help them escape the immune system and/or destroy the intestinal barrier. Certain dietary factors can improve intestinal health, maintain performance and help the animals to cope with a disease challenge. Among these dietary factors, clays have been shown to have beneficial effects.

Dietary clays are used in livestock primarily as mycotoxin binders and as additives that contribute to improve the flow of the feed in bins and feeders, thus reducing problems with caking of feed. However, it has been shown that clays can also reduce diarrhea. In humans, clays have been used to ameliorate diarrhea (Carretero, 2002), however, clays have also been used in the pig industry with some success. Clays clearly reduce the frequency of diarrhea (Song et al., 2012) in pigs and also parallel observations have been made in rats (Gonzales et al., 2004). Clays also enhance intestinal morphology, which can lead to improvement in intestinal health (Xia et al., 2004, 2005). However, little information about the specific mode of action of clays is available. A variety of clays are available, and they probably have different applications and modes of action.

The reduction in diarrhea by dietary clays seems clear, but the mechanisms through which the clays produce this benefit are not. Thus, the objective of this research is to investigate

mechanisms by which clays may reduce diarrhea in pigs and poultry. Of several possible mechanisms our focus is on the strengthening of the intestinal barrier, as it is the most solidly supported by the literature. Further research on the impact of dietary clays on barrier function is needed.

A review of literature regarding defense mechanisms, intestinal infections and dietary clays is provided in Chapter 2. In Chapter 3 we present data on an *E.coli* challenge and how it alters intestinal barrier of pigs in the presence or absence of clays. In Chapter 4, we provide novel data on the effects of clays on performance and barrier function of chicks challenged with *Salmonella enterica* serovar Typhimurium. Chapter 5 provides data on the effects of smectite A on LS174T cells (a human colorectal adenocarcinoma cell line) and the expression of genes related to enhancing intestinal barrier, as well as data on the effect of smectite A on gene product. We conclude with a proposed interpretation of the entire body of work.

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CHAPTER 2

DEFENSE MECHANISMS, INTESTINAL INFECTIONS, AND DIETARY CLAYS: LITERATURE REVIEW[1]

INTRODUCTION

In the food animal industry enteric diseases cause important economic losses in spite of powerful health technologies such as all-in/all-out movements, sanitation, biosecurity, vaccines, and others (Hardy, 2002). In order for the animals to cope with disease there are several non-immunological and immunological defense mechanisms in the gastrointestinal tract involved in responding to the pathogens (Sarker and Gyr, 1992; Mariscalco, 2011).

The non-immunological defenses involve gastric acid, intestinal motility and microflora, lysozyme, pancreatic secretions, bile (Sarker and Gyr, 1992), and the physical barrier of the intestinal epithelial cells including the mucus layer (Schenk and Mueller, 2008). The focus of this review will be on the intestinal epithelial barrier because we hypothesize that one of the mechanisms through which clays provide benefits is strengthening the epithelial barrier.

The immunological defenses are divided into innate and adaptive ones, even though in practice there is much interaction between them (Parkin and Cohen, 2001). The innate response is not specific to a particular pathogen (Alberts et al., 2002) and provides immediate host defense (Parkin and Cohen, 2001). The innate response includes neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins but sometimes the term also includes physical, chemical and microbiological barriers as well (Parkin and Cohen, 2001).

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There is evidence that several dietary factors can help maintain health and growth performance of disease-challenged animals (Perez et al., 2011; Che et al., 2012). Clays have been used in human medicine to ameliorate diarrhea (Carretero, 2002), and they are also used in the pig industry with some success (Schell et al., 1993; Trckova et al., 2009; Song et al., 2012) but their efficacy in chicks challenged with enteric disease has not been reported.

Little information regarding the mode of action of clays in pigs and poultry is available. Thus, the objectives of this literature review are to provide information regarding the use of dietary clays and to explore potential mechanisms through which clays may produce benefits, with primary emphasis on barrier function.

DEFENSE MECHANISMS

The intestinal mucosa is exposed to a great amount of antigens and this poses a challenge to homeostasis maintenance (Schenk and Mueller, 2008). The potential pathogens can be acquired through ingestion, inhalation or contact (Alberts et al., 2002). The adaptive immune system has an important role in protection against disease; however, due to the adaptive system's slow response on first exposure it is the innate immune system that is critical in protecting the host during the first few hours or days of infection (Alberts et al., 2002).

Innate immune system

In order to handle the heterogeneity of the microbes, the innate immune system has pattern recognition receptors (**PRRs**) that recognize pathogen-associated molecular patterns (**PAMPs**), which are highly-conserved structures present in microbial pathogens. A few examples of PAMPs are lipopolysaccharide (**LPS**), bacterial DNA, peptidoglycan, and others (Mariscalco, 2011).

Many cell types are involved in the innate immune response such as neutrophils, macrophages, monocytes, natural killer (**NK**), and dendritic cells; these cells migrate toward the site of infection (Dempsey et al., 2003). In early stages of infection, there is release of specialized proteins called cytokines, from activated macrophages (phagocytic cells) that increase recruitment of neutrophils. Neutrophils are the first cells to migrate from the blood to the site of infection and they are involved with phagocytosis (Parkin and Cohen, 2001).

Cytokines travel short distances to interact with the target cell surface receptors. Cytokines can be detected in serum especially when their production is maximized such as in sepsis. There are pro- and anti-inflammatory cytokines. Examples of pro-inflammatory cytokines include interleukin (**IL**) 6, and, interferon γ (**IFN**-γ). Examples of anti-inflammatory cytokines include transforming growth factor β, and IL10. In some cases multiple cytokines contribute to a common outcome, but each cytokine also has specific effects. For example, IFN-γ activates monocytes, macrophages, and neutrophils, thus enhancing the killing of intracellular organisms. The IFN-γ also induces production of nitric oxide, which is bactericidal, and is very important in helping to cope with Salmonellosis infection (Mariscalco, 2011).

The release of cytokines triggered by infection or inflammation induces the synthesis of acute phase proteins (**APPs**), which are a group of proteins involved in restoration of homeostasis, activation of complement, modulation of the immune response, and limitation of bacterial growth (Gabay and Kushner, 1999; Murata et al., 2004). Examples of APPs include haptoglobin, C-reactive protein, serum amyloid A, ceruloplasmin, fibrinogen, and α-1-acid glycoprotein (**α-1-AGP**) (Murata et al., 2004). After a few hours of infection, the pattern of proteins synthesized in the liver is changed, resulting in an increase of APPs (Gruys et al., 2005).

Intestinal barrier function

The intestinal barrier is composed of the epithelial barrier cells (enterocytes, goblet cells, enteroendocrine and paneth cells), tight junctions, mucus, and associated immune cells (Kim and Ho, 2010; Rescigno, 2011). Stem cells are located in the crypt compartment; paneth cells undergo differentiation and settle at the crypt bottom (Kim and Ho, 2010). Cells that are actively proliferating are located at the base of the crypt and migrate toward the luminal surface as they differentiate and are eventually sloughed off (Evans and Liu, 2008).

- ***Tight junctions***

A single layer of intestinal epithelial cells is the major site of absorption of nutrients as well as the most important barrier between the internal and external environments (Söderholm and Perdue, 2006). The cell membranes control passage of materials through the cells, and close connections between cells that limit paracellular leakage of materials are created by adherens junctions and tight junctions. Adherens junctions provide strong connections that enable closeness of the cells and also a site for communication (Turner, 2009). The tight junctions are more apical than the adherens junctions but require the adherens junctions for their assembly (Turner, 2009). Tight junctions are complex structures that include transmembrane proteins (claudin and occludin), peripheral membrane proteins (zonula occludens) and regulatory molecules (Turner, 2009). The tight junction tightens the lateral intracellular space between adjacent cells, preventing paracellular leakage.

- ***Mucus***

Mucus is the first protection against luminal microbiota (Kim and Ho, 2010) and separates the intestinal lumen from the epithelium (Rescigno, 2011), protecting against foreign insults (Lai et al., 2009), and also against pathogens (Sellers et al., 1988). It can also act as a lubricant to avoid epithelial damage (Sellers et al., 1988). Mucus is present throughout the entire

gastro intestinal tract (Marchiando et al., 2010). The intestinal mucosa is always exposed to several antigens such as food, bacteria, viruses, etc. (Söderholm and Perdue, 2006) and the mucus is thicker where the microbiota are more abundant (Rescigno, 2011). Mucus is constantly being shed, secreted, digested, discarded, and recycled (Lai et al., 2009) and the thickness of the mucus layer depends on the balance among synthesis, secretion and degradation (Kim and Ho, 2010). Microbial proteases and glycosidases are involved in mucus degradation (Kim and Ho, 2010); glycosidases are involved in degrading mucin sugars. Once these terminal sugars are degraded, the oligosaccharides and the protein core of mucin are exposed and mucin can then be easily degraded by microbial proteases (Png et al., 2010). These microbial enzymes compromise the protective function of the mucus especially when the rate of degradation exceeds the rate of synthesis (Macfarlane et al., 2005).

Mucus mesh is composed of mucins combined with other components (such as lipid, ions, protein, cellular debris, and water), most of it (> 90%) being water (Lai et al., 2009). Mucins are large glycoproteins with highly polymeric protein backbones attached to oligosaccharide side-chains that form a gel structure (Kim and Ho, 2010).

- ***Goblet Cells and Their Products***

Goblet cells are specialized epithelial cells that secrete mucins, mainly mucin 2 (**MUC2**) and other products involved in promoting barrier function (Moncada and Chadee, 2002), such as trefoil factor 3 (**TTF3**) and resistin-like molecule beta (**RELMβ**). Goblet cells are located in the small intestine and colon, but reportedly at greater density in the colon (Specian and Oliver, 1991). The population of goblet cells among epithelial cells was reported to increase caudally (4% duodenum – 16% in the distal colon) (Kim and Ho, 2010). There are fewer and smaller

goblet cells in the intestine of a germ-free mouse compared to a conventional mouse (Kim and Ho, 2010).

Goblet cells are present in villi as well as crypts and possess theca containing mucin granules (Kim and Ho, 2010). In *MUC2*-knockout (**KO**) mice there are no identifiable goblet cells even though there is still presence of other products normally produced by goblet cells. However, in *TFF3*-KO mice small goblet cells are identifiable and these indicate the important role of MUC2 in goblet cell morphology (Kim and Ho, 2010).

Krüppel-like factor 4 (***KLF4***) is expressed in the small intestine in gut epithelial cells (Hinnebusch et al., 2004) and is important for development, differentiation and maintenance of normal tissue homeostasis (Evans and Liu, 2008). The KLF4 inhibits proliferation and promotes differentiation and is specifically required for goblet cell differentiation (Evans and Liu, 2008). Inflammation stimulates expression of *KLF4* and that inhibits proliferation and stimulates differentiation (Evans and Liu, 2008).

- ***Mucins***

The glycoprotein structure of mucin has a central protein core surrounded by carbohydrate chains covalently attached to it (Allen and Leonard, 1985), and a terminal sparsely-glycosylated region rich in Cys. There are secreted (MUC2, MUC5AC, MUC5B and MUC6) and membrane bound (MUC1, MUC3, MUC4, MUC 13 and MUC17) mucins. The membrane bound mucins are firmly adherent and tightly bound to the glycocalyx on the luminal side of the enterocytes (Figure 2.1). The secreted mucins are loosely adherent but represent a thicker layer compared to the membrane bound (450 vs. 30um) and are on the luminal side (Kim and Ho, 2010).

The major secreted mucin in the intestine is MUC2. The MUC2 monomer is very large with over 5,000 AA and a molecular weight of 2.5 MDa, and with a central domain rich in Pro, Ser and Thr. These mucins form dimers in the endoplasmic reticulum via intermolecular disulfide bonding between c-terminal cysteine knot domains (Kim and Ho, 2010) and during their transit through the golgi apparatus they became o-glycosylated at Ser and Thr and undergo trimerization via disulfide bonding in the amino terminal region which results in a very large polymer (Kim and Ho, 2010). The secretion of MUC2 is stimulated by several factors including microbes, toxins, inflammatory cytokines and others (Kim and Ho, 2010). Mucin secretion can be associated with increased mucin production but chronic secretion can lead to depletion of goblet cells (Kim and Ho, 2010). During the acute phase of intestinal infections there is an induction of mucin synthesis and secretion but during the chronic phase there may be depletion of goblet cells (Kim and Ho, 2010). Membrane bound mucins are also expressed by intestinal epithelial cells (Kim and Ho, 2010).

Kim and Ho (2010) reviewed the functions of the major goblet cell products. The secreted mucin *MUC2,* as mentioned before, is the major secreted mucin in the small intestine. It provides protection and lubrication and it can also be nutrient source of microbes. The *MUC5AC*, *MUC5B* and *MUC6* are also secretory mucins expressed in gastric and respiratory epithelium. The membrane bound mucins, *MUC1*, *MUC3, MUC17* are protective to the cell surface and involved in epithelial restitution in lungs and small intestine.

Some mucins are pH neutral and some are acidic. Acid mucins are divided into sulfo and sialomucins (Martinez et al., 2010). Acid and neutral mucins can be differentiated by staining with Alcian blue-periodic acid-Schiff (**AB/PAS**) reagent (Yamabayashi, 1987). A stain to differentiate the two types of acid mucins is high iron diamine followed by Alcian Blue (**HID/**

AB) at pH 2.5 (Reid et al., 1989) which presents sulfate as brown-black and carboxyl groups as blue.

- ***Trefoil factors***

The TFF are secreted in the gastrointestinal tract (Thim et al., 2002) and are usually associated with the mucin layer where they have a healing function (Thim, 1997). There are 3 trefoil factors: TFF1, TFF2, and TFF3. The *TFF1* is expressed mainly in the stomach, *TFF2* mainly in the stomach, duodenum, and pancreas, and *TFF3* mainly in the intestine (Thim, 1997). The *TFF3* is almost exclusively expressed by goblet cells. It co-localizes with *MUC2* (Taupin and Podolsky, 2003) and is involved in repair of mucosal epithelial damage in order to maintain barrier function and prevent inflammation.

These are small peptides (6.5 to 12 kDa) which play a significant role in epithelial restitution but they are more effective when acting in conjunction with mucin (Kim and Ho, 2010). The TFF3 (Kim and Ho, 2010) is involved in promoting cell migration (but not proliferation, which is promoted by RELMß).

- ***Resistin like molecule beta***

These are small, Cys-rich secreted proteins that are dimers of 25 kDa (Kim and Ho, 2010). They are produced and secreted mostly in the large intestine (Kim and Ho, 2010).

The expression of *RELMβ* is induced upon bacterial colonization, thus, it is associated with mucosal injury in certain pathological conditions. The RELMβ likely plays a defensive role against nematode intestinal infection (McVay et al., 2006) and is involved in maintenance of colonic epithelial cell barrier function (Hogan et al., 2006). RELMß upregulates *MUC2* transcription and secretion (Kim and Ho, 2010). It is highly secreted by goblet cells of the colon and is secreted in response to bacterial colonization (Fujio et al., 2008). RELMß may have an

immunoregulatory (Hogan et al., 2006) function as it regulates colonic inflammation susceptibility, which is dependent on the mucosal barrier integrity. It participates directly in maintaining the mucosal defense barrier and has a regulatory role in colonic inflammation (Krimi et al., 2008; Kim and Ho, 2010).

INTESTINAL INFECTIONS

Escherichia coli infection

Escherichia coli (***E.coli)*** infection, also known as colibacillosis, is an important cause of illness and death in neonatal and weaned pigs (Francis, 1999) which leads to economic losses in swine production. *E.coli* are gram-negative bacteria, thus they produce LPS. The LPS is composed of 3 different parts: lipid A (hydrophobic lipid moiety), a core oligosaccharide that attaches to lipid A, and the O-antigen polysaccharide (Gyorfy et al., 2012). During infection the bacteria can release LPS from the membrane and LPS can cause effects on enterocytes as well as other cells (Gyorfy et al., 2012). The LPS stimulates activation of the innate immune system (Gyorfy et al., 2012).

There are several strains of *E.coli* but for the purpose of this review we will mention only the group classified as enterotoxigenic *E.coli* (**ETEC**) and that produces the F18 fimbriae (the main type of fimbriae associated with diarrhea in weaned pigs). The ETEC produce fimbrial adhesins, heat-labile (**LT**) and heat-stable (**ST**a and STb) toxins, and Shiga toxin (Francis, 1999). The fimbrial adhesins allow the bacteria to bind to receptors on the enterocytes in the small intestine (Melkebeek et al., 2012). The attachment to enterocytes is required for pathogenesis, as it allows the *E. coli* to secrete its toxins. Shiga toxin is responsible for edema disease in addition to diarrhea (Francis, 1999). The LT, STa and STb induce changes in the enterocytes resulting in

increased loss of water, causing dehydration although by different mechanisms (Zhang et al., 2006). The LT increases cAMP levels causing increase in Cl^- secretion and H_2O secretion and reduction in Na^+ absorption (O'Brien and Holmes, 1996). The STa leads to an increase in cGMP levels causing an increase in Cl^- secretion and H_2O secretion and a reduction in Na^+ and Cl^- absorption (Forte et al., 1992). The STb, on the other hand, stimulates secretion of HCO_3^- from epithelial cells (Weikel and Guerrant, 1985).

Salmonella enterica **infection**

Salmonella enterica serovar Typhimurium (***S. typhimurium***) is also a gram-negative bacterium. *S. typhimurium* has a wide range of hosts such as human, ruminant, avian species and others (Garai et al., 2012). It is an intracellular pathogen; after entering the small intestine, the bacterium crosses the mucous layer and must escape being killed by enzymes or innate defenses, then gains access to the underlying epithelium (Sansonetti, 2004). It mainly colonizes the ileal portion of the small intestine but can also extend into the colon.

Virulence factors include adherence to the microfold cells and a secretory system used to invade host cells (Sansonetti, 2004). The attachment of the bacterium to the host cells consists of several steps that involve several adhesins. Each adhesin is responsible for the attachment to a particular kind of cell depending on the cell receptor. The flagellum is required for the bacterium to reach the host cell as well as to help with adhesion (Garai et al., 2012). The needle-like type III secretory systems on the bacterial cell wall (Garai et al., 2012) allow the bacterium to directly inject toxin effectors into host cells (Sansonetti, 2004).

The use of feed additives in pigs and poultry diets that are able to impede the attachment or invasion of these pathogens is desirable. If clays can enhance the barrier function, that may

reduce the attachment and colonization of these pathogens to intestinal cells, thus reducing the economic losses and morbidity caused by diarrhea in livestock production.

DIETARY CLAYS

Definition

Clays are naturally occurring minerals with particle size < 2.0 μm in diameter. They harden when exposed to heat and their plasticity is affected by their chemical composition. The primary composition is phyllosilicates (from Greek *phyllon*, leaf, and from latin *silic*, flint).

Chemical Structure and Properties

The structure of phyllosilicates is based on tetrahedral sheets of cations (commonly Si^{4+}, Al^{3+}, and Fe^{3+}) and octahedral sheets of cations (commonly Al^{3+}, Fe^{3+}, Mg^{2+}, and Fe^{2+}). The phyllosilicates of clays occur in layers (Bergaya and Lagaly, 2006). These layers are classified into 1:1 (Figure 2.2), 2:1(Figure 2.3), and framework structures (Figure 2.4). The 1:1 layer is a tetrahedral Si sheet bound covalently to an octahedral Al sheet (Meunier, 2003). A few examples are kaolinite, dickite, and nacrite, which belong to the kaolin group (Brigatti et al, 2006). The 2:1 layer is an octahedral sheet (Al, Mg or Al and Mg) between 2 tetrahedral Si sheets (Meunier, 2003). A few examples are montmorillonite, saponite, hectorite, and beidellite, which belong to the smectite group as well as illite, chlorite, and vermiculite (Murray 2007). Zeolite is an example of the framework structure which is also called structure with channels (Meunier, 2003). These are 3-dimensional tetrahedral structures of SiO_4^{4-} and AlO_4^{5-} linked through shared oxygen atoms (Song et al., 2012).

The structure and composition of clays that are commercialized are very different even though all are composed of tetrahedral and octahedral sheets as their building blocks (Murray,

2007). Their structure and composition determine their physical and chemical properties. For instance, smectites and illite have spaces between layers that can expand to accommodate water and cations (Murray, 2007), allowing exceptional water adsorption; they are able to adsorb up to half of their mass in water (Schoonheydt and Johnston, 2006). Montmorillonite (**MMT**) has a charge imbalance due to replacement of Al^{3+} for Si^{4+} in the tetrahedral layer and Mg^{2+} or Zn^{2+} for Al^{3+} in the octahedral layer, resulting in a negative charge at the surface (Xia et al., 2004a) which results in strong adsorptive power, and high stability (Xia et al., 2004a). Smectites have medium to high cation exchange capacity, high surface area, high adsorption capacity, and high viscosity (Murray, 2007). Kaolin minerals (kaolinite, dickite, nacrite, and halloysite) have minimal net charge and properties such as very low cation exchange capacity, low surface area, and low adsorption capacity (Murray, 2007). Zeolites have three-dimensional channel structures that can trap molecules according to their dimensions (Shurson et al., 1984). Exchange of cations and water takes place within the three-dimensional channel structures present in zeolites. They have a net negative charge (Czurda, 2006), high exchange capacity for certain cations (NH_4^+, Pb^{2+}, Cd^{2+}, Sr^{2+}) with their selectivity a function of the pore size, and capacity to adsorb contaminants (Bergaya et al., 2006). Zeolite and smectite hydration/dehydration are continuous and reversible (Bish, 2006).

Toxicity of clays

There is concern about health hazards experienced by workers in clay mines, largely attributed to inhalation. In lungs, clays may cause cancer, or pneumoconiosis, but the results of several reviews (in humans) according to Carretero et al. (2006), although contradictory, suggest that most of the toxicity is due to other substances often found along with clays, such as quartz

and asbestos. Both the dose and the time of exposure are important factors but the quantitative relationships to toxic effects are unclear (Carretero et al., 2006).

One type of clay, a hydrated sodium calcium aluminosilicate (**HSCAS**) with a 2:1 layer structure, has been proven to have no detrimental effect when fed to broilers or laying hens at dietary concentrations that were 8 times higher (up to 2% of the diet) than the level often recommended (0.25%). The HSCAS had no detrimental effect on performance of broilers from hatch to 3 wks of age or laying hens (Miles and Henry 2007a,b,c)**.** This has not been proven for all the clays but the USDA and FDA consider clay-based adsorbents safe as dietary flow agents when used at 2% to 5% of the diet (Miles and Henry 2007c).

The special case of modified clays

Clays can be modified by addition of an excess of specific cations that may cause them to have antimicrobial and other effects, which can potentially improve growth performance and health. The modification of the natural structure of clays is achieved in practice via acid activation (Hu and Xia, 2006), cation exchange, and heat treatment (Haydel et al., 2008). These modified clays are considered separately here because their effects may be from the added cations rather than from the clays themselves.

Inclusion of pharmacological levels of Cu in pig diets has been shown to have beneficial effects on growth performance (Cromwell, 2001). Feeding a mixture of clays and Cu^{2+} increased ADG and G:F, and decreased incidence of diarrhea in pigs or broiler chicks (Xia et al., 2004a,b; Xia et al., 2005). The Cu-MMT reduced viable counts of *E.coli* and *Clostridia* in the small intestine (**SI**) and cecum of broiler chicks, but this effect was not present when MMT without Cu was fed (Xia et al., 2004b). *In vitro*, modified clays that had Cu^{2+} addition had an antibacterial effect on *E.coli* F4 (Xia et al., 2004b; Hu and Xia, 2006) as well as on *Salmonella*

cholerasuis (Tong et al., 2005). The effect is probably due to a slow release of Cu^{2+} into the broth. Antimicrobial effects of these modified clays may lead to an improvement in growth performance.

An antimicrobial effect is also attributed to the French Green Clays (Fe-rich smectite and illite) when enriched with Mg^{2+} or K^{+} but those effects were not demonstrated when French Green Clays were enriched with Ca^{2+} (Haydel et al., 2008). Another modified clay, Ca-bentonite, improved ADG and ADFI of piglets when fed in an aflatoxin-contaminated diet (Schell et al., 1993), an effect that may have resulted from binding of the aflatoxins to the clay. Synthetic zeolite had a negative effect on growth performance of pigs (Shurson et al., 1984).

POTENTIAL BENEFITS OF DIETARY CLAYS

Growth performance

When fed to pigs, kaolin has either improved ADG (Trckova et al., 2009) or had no detectable effect on it. When fed to broilers, kaolin had no detectable effect on performance (Sellers et al., 1980). Experimental challenges with ETEC did not alter the effect of kaolin on growth performance of pigs (Trckova et al., 2009; Song et al., 2012). The beneficial effect of kaolin on ADG of pigs was found with a dietary inclusion rate of 1% (Trckova et al., 2009), whereas the lack of response was to lower (0.3%; Song et al., 2012) or high (1 and 3%; Rivera et al., 1978) concentrations.

Smectite has been shown to improve growth performance when fed to unchallenged weanling pigs at 0.3 or 0.6% of the diet (Song et al., 2012). Smectite has also shown to improve feed efficiency when fed to laying hens (Sellers et al., 1980). The benefit did not occur in pigs challenged with ETEC or when fed to unchallenged broilers (Sellers et al., 1980).

The effect of zeolites on growth performance is variable. Growth rate was increased by feeding zeolite at 2% of the diet during the weaning period (Papaioannou et al., 2004) or from weaning to market (Papaioannou et al., 2004; Alexopoulos et al., 2007). However, no benefit was found from lower levels, including 0.5% of the diet for growing pigs for 6 wks (Shurson et al., 1984) or 0.3% of the diet for weaned pigs (Song et al., 2012) either challenged or not with ETEC. Zeolite at 0.3% of the diet fed to growing pigs had no effect on growth rate (Shurson et al., 1984). It has been suggested that the kind of zeolite may also influence its effects (Shurson et al., 1984).

There is, therefore, inconsistency in the effects of natural clays on growth performance of pigs. Several factors may contribute to that such as type of clays, its characteristics such as molecular weight, crystalinity, purity etc, (Shurson et al., 1984; Papaioannou et al., 2004), particle size, inclusion level (Alexopoulos, et al., 2007), and geographical source (Xia et al, 2004a). Moreover, the age of the pig and duration of the supplementation may influence the response to dietary clays. The weaning age reported for young pig experiments ranged from 21 to 35 d (Rivera et al., 1978; Schell et al., 1993; Alexopoulos et al., 2007; Trckova et al., 2009; Song et al., 2012) and other authors studied clay supplementation in growing and finishing phases (Shurson et al., 1984; Alexopoulos et al., 2007). Breed or genetics may also be a source of variation in observed responses to clay supplementation, as Pond et al. (1981) observed that breed and dietary zeolite interacted in affecting growth rate.

Enteric disease

We have previously observed in our lab (Song et al., 2012) that dietary clays (smectite, kaolinite, zeolite and all possible combinations at 0.3% inclusion level in the diet) clearly reduced both diarrhea score and the frequency of diarrhea in young pigs challenged with a

pathogenic *E. coli*. Feeding the various clay treatments to the challenged pigs resulted in a 59 to 85% reduction in frequency of diarrhea. The clays also reduced the population of β-hemolytic coliforms in the challenged pigs (Song et al., 2012). This ability of clays to reduce diarrhea in pigs has also been shown by Trckova et al. (2009), and parallel observations have been made in humans (Carretero, 2002) and rats (Gonzales et al., 2004). This response to dietary clays appears, on the basis of limited data, to be more consistent than the response of growth performance.

Caution should be used when extrapolating disease-challenge data from these experiments to practical environments because each experiment uses only one challenge organism, all pigs are infected at the same time, and the environment is ideal.

Possible mechanisms of action

The reduction in diarrhea by dietary clays seems clear, but the mechanisms through which the clays produce this benefit are not. Several possible mechanisms are suggested in the literature, but we focus here on only a few of the more prominent ones, including toxin adsorption, antimicrobial effects, and strengthening of the intestinal barrier.

- **Toxin adsorption**

Mycotoxins are secondary metabolites produced by fungi and are toxic to animals. These toxic compounds (e.g., aflatoxin, alkaloids, fumonisin, orchratoxin, vomitoxin, zearalenone) may be present on cereal grains (Lindemann et al., 1993; Ledoux and Rottinghaus, 2000). Mycotoxins are harmful to animals in regard to health, growth performance, and overall production. The mycotoxin adsorption effect of clays is well known (Phillips et al., 1988; Harper et al., 2010; Zhao et al., 2010) but it is not the subject of this review.

In addition to mycotoxins, clays may bind other toxins as shown by *in vitro* studies (Ramu et al., 1997) where Na-bentonite adsorbed and inactivated LT enterotoxins of *E. coli* and

the cholera enterotoxins of *Vibrio cholerae*. Kaolinite and HSCAS can adsorb bovine rotavirus *in vitro* (Clark et al., 1998) but without removing the virus infectivity; thus the virus is adsorbed but still has its ability to establish an infection.

- **Antimicrobial effects**

As mentioned earlier, clays modified by addition of copper or other cations may have antimicrobial properties (e.g. Xia et al., 2004b; Tong et al., 2005). However, the literature appears to show no compelling evidence that natural clays without high content of specific cations are antimicrobial.

- **Strengthening the intestinal barrier**

Some clays may enhance intestinal morphology, which is associated with enteric health. Feeding 0.15% of MMT to pigs (Xia et al., 2005) or broilers (Xia et al., 2004a) increased villus height and VH:CD in jejunum.

Feeding clays may protect the intestine against damage. When feeding 1% kaolin to weaned pigs challenged with a pathogenic *E.coli*, Trckova et al. (2009) observed a reduction in microscopic lesions in the intestines. Feeding smectite to rats with hapten-induced (trinitrobenzene sulphonic acid) colitis reduced intestinal macroscopic damage (Gonzales et al., 2004).

Kaolinite interacts with the intestinal mucosa of rats, altering the mucosal morphology and embedding itself in the brush-border layer (Reichardt et al., 2009). Electron microscopy revealed that kaolinite particles covered the villi in the jejunum. These effects were seen after the rats consumed kaolinite *ad libitum* for 28 d, but not for 7 d. Similarly, smectite interacts with the glycoproteins in mucus, increasing viscosity and spreading across the surface of the intestinal mucosa (Droy-Lefaix et al., 1985; Droy-Lefaix, 1987). If clays interact chemically with mucins,

the specific types of bonds that are involved in this interaction have not been identified. Both clays and glycoprotein mucins have predominantly negative charges, which makes it unlikely that the interaction is a simple anion-cation reaction.

Feeding smectite to rats with hapten-induced colitis increased colonic MUC2 levels and reduced the colonic level of the pro-inflammatory cytokine IL-1β at the peak of infection (Gonzales et al., 2004). The increase in MUC2 levels may strengthen the barrier function. Perhaps the strengthened barrier provides protection from invasion, and the lesser invasion reduces inflammation as indicated by the lower IL-1β concentration.

The literature provides more support for barrier function as a mechanism for reducing diarrhea than for the other potential mechanisms identified. Further research on the impact of dietary clays on barrier function is needed.

SUMMARY AND CONCLUSIONS

Enteric diseases are an important cause of economic losses in the animal industry. In order to cope with disease, the gastrointestinal tract uses immunological and non-immunological mechanisms of defense. The innate system provides immediate response but it is not specific. The adaptive immune system provides specific response but it is not immediate during the first exposure to a pathogen. Both the innate and adaptive immune systems act together. The physical intestinal barrier provides protection; it includes tight junctions and mucus secretion.

In the livestock industry, animals are susceptible to enteric infections. *E.coli* and *Salmonella* are two pathogens known to cause enteric infection in pigs and poultry. These pathogens, depending on several conditions such as dose of pathogen, age of the host, immune status of the host and others, can overcome the immune system and damage the intestinal barrier

Consequently, the use of selected feed ingredients is beneficial in order to reduce the negative impacts that *Salmonella*, *E.coli* and other enteric pathogens can cause.

There are several dietary factors that can help to maintain or improve health of disease challenged animals. Clays have been used in human practice to ameliorate diarrhea but they are also used in the pig industry with some success. Dietary clays can improve ADG, intestinal morphology and reduce diarrhea. However, little is known about the mechanism through which dietary clays produce these benefits.

In practice, clays are mainly used as mycotoxin binders and flowability agents in poultry and pig diets. There are a variety of clays available and they probably have different applications and modes of action. Previous experiments from our lab reported that smectite, zeolite and kaolinite at 0.3% (as well as their combination) of the diet to pigs challenged with a pathogenic *E.coli* reduced diarrhea in those pigs. However, as mentioned before, the mode of action of clays in reducing diarrhea is not well understood. Little information regarding dietary clays' effects in poultry diets related to intestinal health as well as the mode of action of clays in pigs and poultry is available. It is necessary to investigate how clays reduce diarrhea. Therefore, strategically-designed research on the effects of dietary clays on the intestines may enhance the knowledge and opportunities of how to prevent or reduce intestinal infection caused by *E.coli* in pigs or *S. typhimurium* in chicks and improve their intestinal health.

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FIGURES

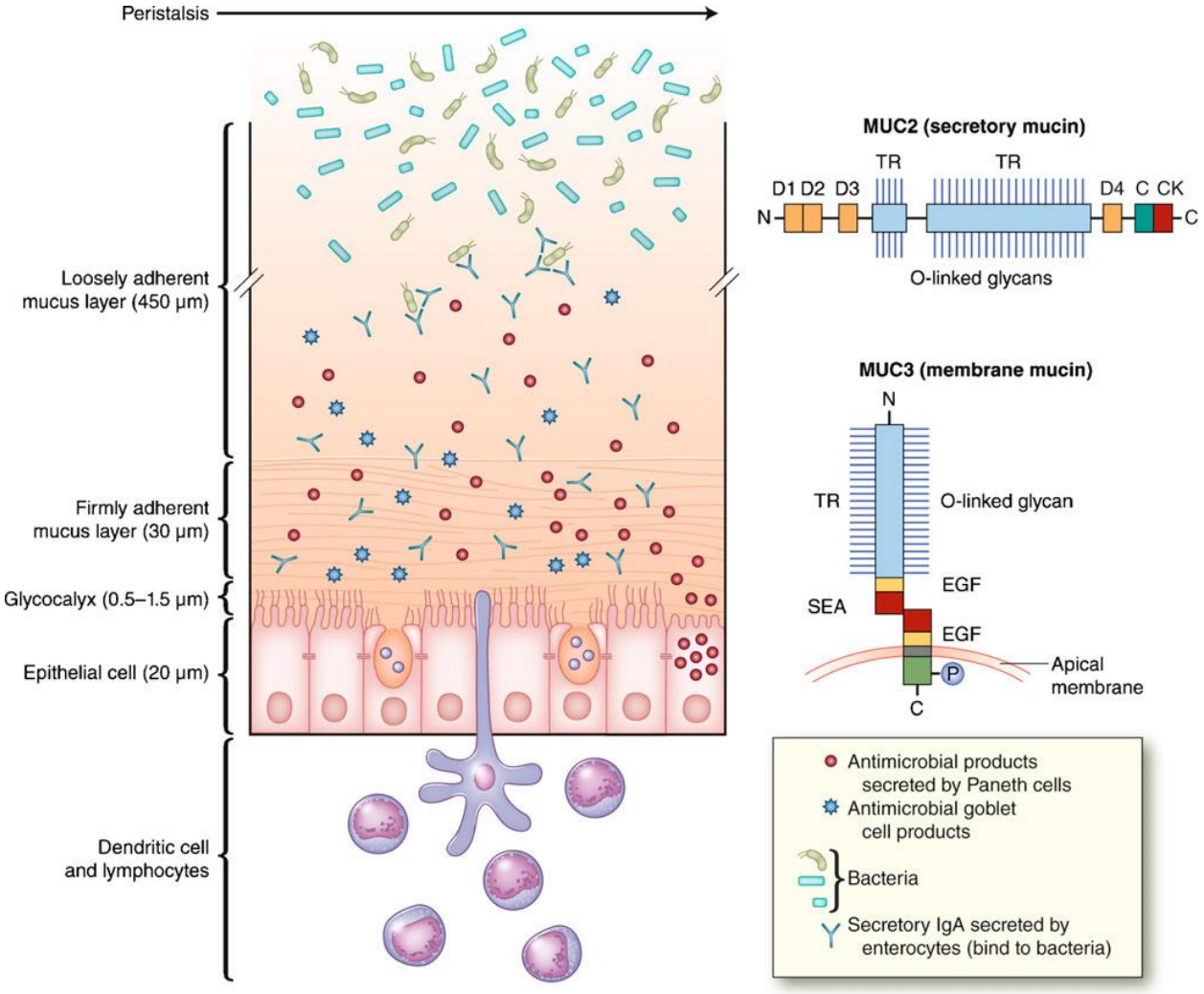

Figure 2.1. A schematic representation of two mucus layers overlying the epithelial cell surface shown (left) and the domain structures of secretory (MUC2) and membrane-bound (MUC3) mucins shown (right). Intestinal epithelial cell surface is covered by two mucus layers (inner, firmly adherent layer and outer, loosely adherent layer) consisting largely of MUC2 mucin network produced by the goblet cells and other host defense molecules produced by goblet cells, Paneth cells, and absorptive enterocytes. Microbes are associated with the outer, loosely adherent mucus layer, but are absent in the inner, firmly adherent mucus layer. Epithelial cell surface is covered by glycocalyx, which consists of membrane-bound mucins (MUC3 and MUC17 in the small intestine) and other membrane glycoproteins. The measurements shown are for the rat ileum. The domain structure of MUC2 monomer shows central tandem repeat (TR) regions rich in proline, threonine, and serine (PTS domain), to which many oligosaccharide side chains (O-linked glycan) are linked, and four von Willebrand factor D domains flanking the tandem repeat (PTS) domains and C-terminal cysteine knot (CK) domain, which is involved in initial MUC2 dimerization. The domain structure of MUC3 mucin shows that it consists of two subunits, one extracellular and one membrane-bound. The extracellular subunit consists of a glycosylated tandem repeat (PTS) domain and two epidermal growth factor (EGF)—like domains separated by sperm protein, enterokinase, and agrin (SEA) motif (a proteolytic cleavage site during biosynthesis) and a membrane-bound subunit that consist of membrane-spanning domain and a cytoplasmic tail with potential phosphorylation (P) sites. Reprinted from Kim and Ho (2010). Reprinted from Current Gastroenterology Reports; Kim, Y. S., and S. B. Ho; Intestinal goblet cells and mucins in health and disease: recent insights and progress; pages 319-330; copyright 2010, with permission from Springer.

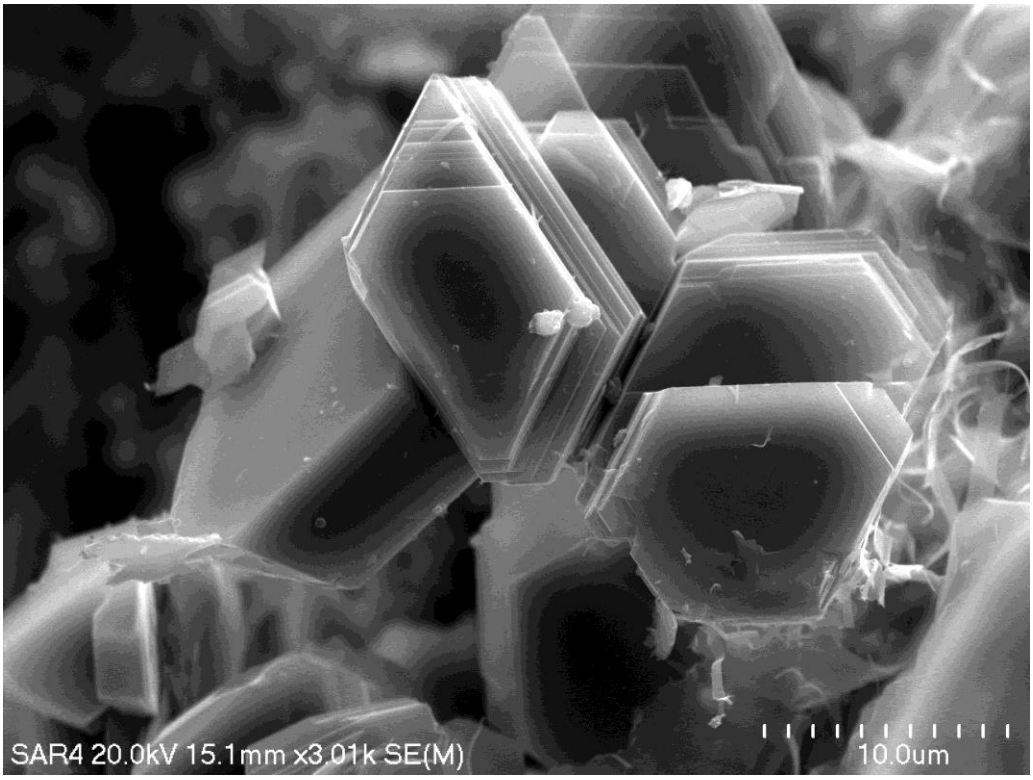

Figure 2.2. Kaolinite. Dimensions: Field of view approx. 40 microns. "Image reproduced from the 'Images of Clay Archive' of the Mineralogical Society of Great Britain & Ireland and The Clay Minerals Society (www.minersoc.org/gallery.php?id=2.").

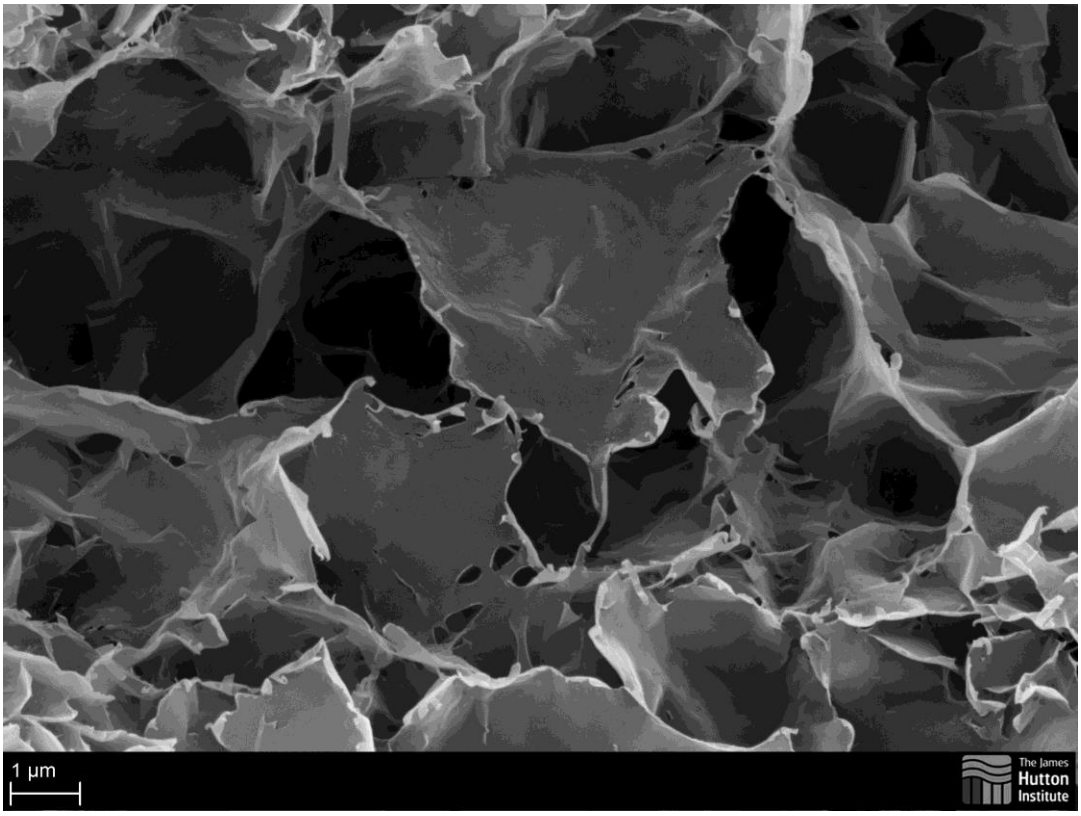

Figure 2.3. Dioctahedral pore-lining smectite. Dimensions: ~15 µm wide. "Image reproduced from the 'Images of Clay Archive' of the Mineralogical Society of Great Britain & Ireland and The Clay Minerals Society (www.minersoc.org/gallery.php?id=2.")

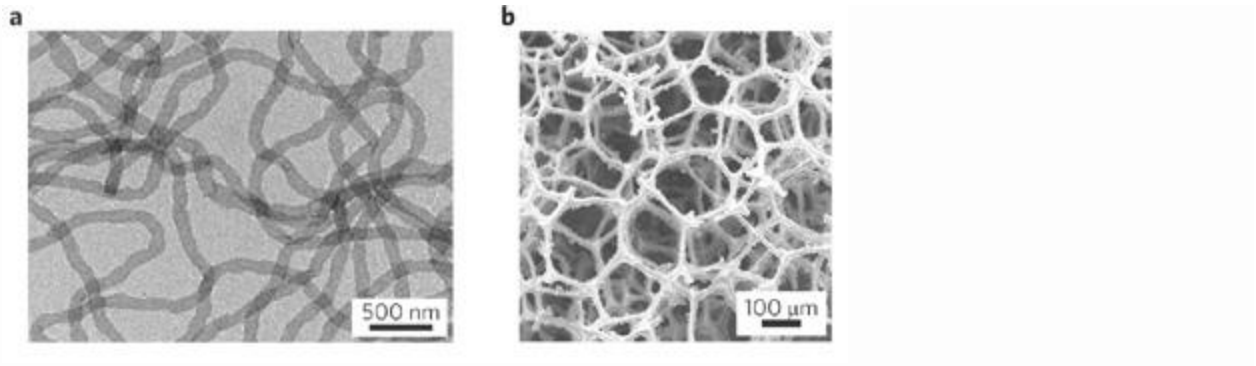

Figure 2.4. Use of self-organized media. TEM image showing SiO_2–polymer nanotubes formed by myelination of poly(ethylene oxide)-*b*-poly(1,2-butylene oxide)–tetraethoxysilane gel particles in water (**a**), and scanning electron microscopy (SEM) image showing a polyhedral framework of zeolite and SiO_2 nanoparticles produced by microphase separation and spatial patterning within the interstitial voids of a dextran-derived foam of CO_2 gas bubbles (**b**). Reprinted from Mann (2009). Reprinted from Nature Materials; S. Mann; Self-assembly and transformation of hybrid nano-objects and nanostructures under equilibrium and non-equilibrium conditions; pages 781-792; copyright 2009; with permission from Nature Publishing Group, U.S.A.

CHAPTER 3

ESCHERICHIA COLI CHALLENGE AND ONE TYPE OF SMECTITE ALTER INTESTINAL BARRIER OF PIGS

ABSTRACT: An experiment was conducted to determine how an *E. coli* challenge and dietary clays affect the intestinal barrier of pigs. Two groups of 32 pigs (initial BW: 6.9 ± 1.0 kg) were distributed in a 2 × 4 factorial arrangement of a randomized complete block design (2 challenge treatments: sham or *E. coli*, and 4 dietary treatments: control, 0.3% smectite A, 0.3% smectite B and 0.3% zeolite), with 8 replicates total. Diarrhea score, growth performance, bacterial translocation from intestinal lumen to lymph nodes, intestinal morphology, relative amounts of sulfo and sialo mucins, and goblet cell size and number were measured. The *E.coli* challenge reduced performance, increased bacterial translocation from the intestinal lumen to the lymph nodes, increased ileal crypt depth, and increased goblet cell size and number in the ileum. One of the clays (smectite A) tended to increase goblet cell size in ileum, which may indicate enhanced protection. In conclusion, *E. coli* infection destroys intestinal barrier integrity but dietary clays may enhance it.

Keywords: *E. coli*, barrier function, pigs, smectite, zeolite

INTRODUCTION

Weaning is a stressful period for piglets due to environmental, social and nutritional changes. During this period, pigs are also vulnerable because of their immature immune and digestive systems (Pluske et al., 2002). The stress may result in depressed feed intake which may lead to poor performance and changes in the intestinal structure and microbiota, thus increasing

the susceptibility of pigs to enteric diseases (Pluske et al., 1997). Post-weaning diarrhea caused by *Escherichia coli* is a common enteric disease in weaned pigs; it causes economic losses due to mortality, morbidity, decreased growth performance and cost of medication (Fairbrother et al., 2005). Diarrhea also impairs nutrient absorption, increases permeability in the intestine, decreases tight junction integrity, increases paracellular movements of molecules and increases infection (Zhu et al., 2012). Among a large number of potential mechanisms are mucosal injury, villous atrophy, increased mast cell number, and reduction in numbers of lymphocytes subsets ($CD8^+$ T and $CD4^+$ T) in jejunum and ileum (Dean and Kenny, 2004; Zhu et al., 2012).

Antibiotics suppress growth of certain microorganisms and are widely used as growth promoters in the swine industry (Cromwell, 2002). However, concern over their potential contribution to antibiotic resistance in bacteria infecting humans has led to tightening restrictions on antibiotic use in animals, including cessation of their use as growth promoters in Denmark in May 1995 (WHO, 2002) and elsewhere more recently. The resulting reduction of growth performance and increase in the morbidity in nursery pigs in Denmark indicate the need for prophylaxis (WHO, 2002). Therefore, it is important to find other reliable strategies to maintain pig health. Among several alternatives, clays have shown promise (Song et al., 2012).

Clays have been used in human medicine to ameliorate diarrhea (Carretero, 2002), and they are also used in the pig industry with some success (Schell et al., 1993; Trckova et al., 2009; Song et al., 2012). In the livestock industry, clays are used mainly as mycotoxin binders and as additives that contribute to improve the flow of the feed in bins and feeders, reducing problems with caking of feed. Clays have not been shown to consistently alter growth performance (Shurson et al., 1984; Xia et al., 2004a,b). Several types of clays are available and they appear to have different applications and modes of action. Clays with both the 1:1 layer structure (e.g.

kaolinite) and the 2:1 layer structure (e.g. smectite) have positive effects on gastrointestinal health of the animals (Droy-Lefaix, 1987; Gonzales et al., 2004). Song et al. (2012) reported that, when pigs were challenged with a pathogenic *E. coli*, feeding dietary clays including smectite, zeolite, kaolinite or combinations of them at 0.3% of the diet reduced diarrhea.

Knowledge of the mechanisms through which clays specifically improve gastrointestinal health is lacking, but there are indications (Droy-Lefaix, 1987; Gonzales et al., 2004) that clays may strengthen the intestinal barrier. Our objectives were to determine the effects of a pathogenic *E. coli* challenge and of dietary clays on the intestinal barrier of pigs.

MATERIALS AND METHODS

Animals, Experimental Design and Diets

The Institute of Animal Care and Use Committee of the University of Illinois reviewed and approved the animal care procedures for this experiment. Two groups of 32 weanling pigs each (about 21d old; initial BW: 6.9 ± 1.0 kg) were obtained from the Swine Research Center of the University of Illinois. Pigs were housed in disease-containment chambers of the Edward R. Madigan Laboratory building at the University of Illinois at Urbana-Champaign from weaning to about 35 d of age. Pigs had 6 d of adaptation period before challenge. There were a total of 32 individual pens, 4 in each of 8 chambers in each suite. There were 2 suites that were used for either challenged or unchallenged pigs and in each suite, 4 chambers in each suite were used. The treatments were arranged in a 2×4 factorial design (without or with *E. coli* challenge and 4 dietary treatments: control, and 0.3% of 3 different clays added to the control diet: smectite A (**SMA**), smectite B (**SMB**) and zeolite (**ZEO**)). The enterotoxigenic (**ETEC**) *E. coli* used for the challenge was isolated from a field disease outbreak, (isolate number UI-VDL 05-27242). It is

an F-18 fimbria+ *E.coli* strain that produces the heat-labile toxin, heat-stable toxin b, and Shiga-like toxin-2 (Perez-Mendoza, 2010). The pigs were orally inoculated with *E.coli* (10^{10} cfu per 3 mL dose) in PBS daily for 3 d continuously to cause mild diarrhea (Perez-Mendoza, 2010). The unchallenged treatment (sham) received a 3 mL dose of PBS daily for 3 d. Both inoculations were given orally beginning 6 d after weaning (d 0). Personnel conducting the experiment were blind to the dietary treatments.

The complex nursery basal diet (Song et al., 2012) was formulated to meet or exceed NRC (1998) estimates of requirements of weanling pigs (Table 3.1). All the other experimental diets were made from the basal and the addition of 0.3% of each dietary clay. It did not include spray-dried plasma, antibiotics, or zinc oxide to avoid their antibacterial or physiological effects. The experimental diets were introduced at weaning (d -6).

Feeding and Sample Collection

Pigs and feeders were weighed on the day of weaning (d -6), the day of the first inoculation (d 0), and d 5, for calculation of ADG, ADFI, and G:F. Diarrhea score was assessed visually with a score from 1 to 5 (1 = normal feces, 2 = moist feces, 3 = mild diarrhea, 4 = severe diarrhea, and 5 = watery diarrhea) daily from d 0 by 1 scorer who was blind to the dietary treatments. Frequency of diarrhea was calculated by counting pig days with diarrhea score of 3 or higher.

The standard *E.coli* vaccine was withheld from the dams of the pigs used in this experiment, as were all routine treatments of the piglets with antibiotics. Prior to weaning, fecal samples of the sows from which we obtained the piglets for this experiment were collected to verify if they were negative for β-hemolytic coliforms by plating on blood and McConkey agars. Plates were incubated at 37 °C and 5% CO_2 for 24 h before reading. Populations of both total

coliforms and β-hemolytic coliforms on blood agar were assessed visually. In the present study β-hemolytic coliforms were detected in the sow feces but they were not the pathogenic *E. coli* we used.

One-half of the pigs (16 from the challenged group (4 from each dietary treatment) and 16 from the sham group (4 from each dietary treatment)) were euthanized on d 5 post inoculation (**PI**) and the remainder on d 6 PI. Prior to euthanasia, pigs were anesthetized by intramuscular injection of a 1-mL combination of telazol, ketamine, and xylazine (2:1:1) per 23 kg of body weight. The final mixture contained 100 mg telazol, 50 mg ketamine, and 50 mg xylazine in 1 mL (Fort Dodge Animal Health, For Dodge, IA). After anesthesia, pigs were euthanized by intracardiac injection of 78 mg sodium pentobarbital per 1 kg of BW (Fort Dodge Animal Health, For Dodge, IA).

Mesenteric lymph nodes were aseptically collected then pooled within pig, ground, diluted and plated in brain heart infusion agar and the results were expressed as CFU per g of lymph node (Swildens et al., 2004).

Three-cm samples of ileum and colon were collected and cut with scissors longitudinally in the mesenteric border. Tissues were gently washed in buffered saline then fixed in Carnoy's solution for 2-3 h. Subsequently tissue samples were placed in 100% ethanol, 95% ethanol, and 70% ethanol for 30 min each and maintained in 70% ethanol until the staining process. The fixed intestinal tissues were embedded in paraffin, sectioned at 5 μm and stained with high iron diamine (**HID**) and alcian blue (**AB**), pH 2.5, as previously described (Deplancke et al., 2000).

Sample Processing and Analysis

After staining, the slides were scanned by NanoZoomer Digital Pathology System (Hamamatsu Co., Bridgewater, NJ), and the measurements were conducted in NanoZoomer

Digital Pathology Image Program (Hamamatsu Co., Bridgewater, NJ). Measurements included villus height, crypt depth, and the cross-sectional area of sulfo- (stained brown) and sialomucin (stained blue). The measurements for villus height and crypt depth were performed on 10 well-oriented villi (Fasina et al., 2010) scanned at 40× resolution.

The total number of goblet cells per villus was counted and NDP.view software was used to measure the cross-sectional area (μm^2) of individual goblet cells. The measurements were performed in 3 well-oriented villi scanned at 40x resolution.

Data were analyzed using the Proc Mixed procedure (SAS Inst., Cary, NC). Pig was the experimental unit. The statistical model included effects of *E. coli* challenge, diet, and their interaction as fixed effects and group as a random effect. Specific contrasts were used to test comparisons between the control and the clay treatments collectively within each challenge group. In addition, differences among the clay treatments within each challenge group were tested by pair-wise comparisons when the overall main effect or the diet x challenge interaction was significant. The χ^2 test was used for the frequency of diarrhea. The α levels of 0.05 and between 0.05 and 0.10 were used for determination of significance and tendency, respectively, among means.

RESULTS AND DISCUSSION

After challenge, fecal samples were collected from pigs from both groups (sham and challenge) and it was observed that both groups of pigs carried β-hemolytic *E.coli*. Subsequent PCR analysis showed that the sham pigs carried *E. coli* that produced cytotoxic necrotizing factor. These results indicate that the sham animals had pathogenic organisms and that the challenged animals could have other pathogenic organisms besides the challenge one, so the model represents a multiple infection rather than an uncomplicated single-pathogen challenge.

The *E. coli* challenge was successful as it increased diarrhea score moderately from d 3 to 5 (Table 3.2) and reduced ADG from d 0 to 5 PI (Table 3.3), consistent with the results of Song et al. (2012). The diarrhea scores were low during the first days after challenge, apparently reflecting a lag period after the inoculation before the clinical signs appeared (Table 3.2). During this period the challenged pigs actually had lower diarrhea scores ($P < 0.05$) than did the sham-challenged ones. During the active disease, from d 3 to 5 PI, the challenged animals had greater diarrhea score than the non-challenged animals ($P < 0.05$), as expected (Table 3.3).

There were no dietary effects on either diarrhea scores (Table 3.2) or growth performance (Table 3.3). The lack of beneficial effects of clays is in contrast to our earlier results (Song et al., 2012). The pigs in this experiment were euthanized at around the peak of disease (d 5 and 6 PI) in order to measure physiological effects of the *E. coli* challenge and the clays at that time. Therefore, diarrhea was assessed for only a short time, with the critical period being d 3-5 PI.

The *E. coli* challenge clearly increased bacterial translocation from the lumen to the lymph nodes but the dietary treatments did not detectably alter it (Table 3.4). To our knowledge, bacterial translocation from the intestinal lumen to the mesenteric lymph nodes has not been reported for pigs challenged with a pathogenic *E. coli* strain. Chicks infected with *Eimeria acervulina*, *E. maxima*, and *Clostridium perfringes* exhibited increased bacterial translocation from intestinal lumen to the spleen when compared with control birds (Collier et al., 2008) indicating that enteric infections reduce the integrity of the intestinal barrier. The increased bacterial translocation caused by *E. coli* in the present study (Table 3.4) indicates that the infection reduced the effectiveness of the intestinal barrier, which was expected.

We did not detect any effect of clays or challenge on intestinal morphology (Table 3.5) except for a tendency ($P = 0.07$) for the effects of clays in increasing VH:CD in the *E.coli*

challenged pigs.Mucins can be acidic or neutral. Acidic mucins are comprised of sulfo- and sialomucins. The body often reacts to infection by increasing the secretion of sulfomucins (Seko et al., 2005) as a protective mechanism; the present data do not show that response (Table 3.6). The present results do not show effects of either infection or dietary clays on the relative amount of sulfo- and sialomucins within goblet cells (Table 3.6).

Goblet cells in the intestine produce mucins, the proteins that comprise the bulk of the mucus layer which acts as the first line of defense against enteric infections (Forder et al., 2007). The present results show that the *E. coli* challenge increased both the number and size of goblet cells in the ileum (Table 3.7), consistent with an increase in mucin secretion in response to pathogenic bacteria or intestinal microbes that has been previously reported (Collier et al., 2008; Deplancke and Gaskins, 2001; Fasina et al., 2010). Perhaps the increased mucin production is a protective response. One of the clays (SMA) tended to increase goblet cell size in the ileum (P = 0.07). There was a trend (P = 0.06) for an interaction between diet and challenge on ileal goblet cell number in which one clay (SMB) increased the number of goblet cells in challenged pigs only. There was a diet effect on goblet cell size in the colon (Table 3.7) in which the clays generally increased cell size, mostly in the sham group. These modest increases in cell size and number during the acute phase of the infection when clays were fed may reflect enhanced protection and may at least partially explain the reduction in diarrhea observed previously in pigs (Song et al., 2012) and children (Szajewska et al., 2006).

One of our previous experiments demonstrated that clays reduced diarrhea during d 3-6 PI (Song et al., 2012); the other showed only a trend during d 3-6 PI but clearer effects later (Song et al., 2012). The benefits of clays in reducing diarrhea that we reported (Song et al., 2012) extend to humans, as a meta-analysis of 9 studies showed that children with acute gastroenteritis

consistently had lower duration of diarrhea when treated with smectite along with re-hydration compared with placebo group without smectite (Szajewska et al., 2006). Therefore, it is not clear if the lack of benefit of clays in reducing diarrhea observed in the present experiment indicates that the clays were not beneficial in this case or if this shorter experiment was not sensitive enough to display the effect.

A minor background infection with a wild strain of β-hemolytic *E. coli* that produces the cytotoxic necrotizing factor occurred in some of the challenged and unchallenged pigs in this experiment. Cytotoxic necrotizing factor is produced by 40% of pathogenic *E. coli* strains involved in urinary tract infections and 5-30% of those involved in diarrheic infections (Hofman et al., 2000); it increases adherence of the pathogen to epithelial cells. The impact of infection with this wild strain on the response to the challenge strain is unclear, but if clays provide protection from diarrhea by strengthening the mucus barrier, they should provide similar protection from both of these strains of *E. coli*.

Weaning triggers a reduction in villus height and in the villus height:crypt depth ratio, caused at least partially by interruption of voluntary feed intake (Pluske et al., 1996), and restoration of villus height may be important for health and growth performance of the pig. In the present study, the challenge increased crypt depth and tended to reduce the villus height:crypt depth ratio (VH:CD; Table 3.5) as shown previously (Perez-Mendoza, 2010). These effects of disease may exacerbate the detrimental impact of weaning on pig health and growth. Our observed values for the sham group are smaller than those previously reported (Perez-Mendoza, 2010). We did not observe beneficial effects of small amounts of dietary clays on villus height as reported by others. For example, montmorillonite increased villus height and villus height: crypt depth ratio in jejunum when fed to weanling pigs at 0.15% of the diet (Xia et al., 2004a). Similar

results were obtained in broiler chickens. Xia et al., (2004b); Xu et al., (2003) and Ma and Guo, (2008) reported that feeding 0.1%, or 0.2% montmorillonite increased villus height and reduced crypt depth in the duodenum and jejunum.

CONCLUSIONS

The present results provide novel information regarding the physiological responses in the intestinal barrier of pigs to a challenge with a pathogenic *E.coli* strain. The clinical benefits of clays in the face of enteric infections that we observed in previous experiments with pigs did not occur in this shorter experiment, but it is unclear whether they may have appeared if the experiment had been longer. Both the infection and the clays altered goblet cell size and number.

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TABLES

Table 3.1. Ingredient composition of experimental control diet (as-fed basis)

Ingredient, %	Control diet
Corn, ground	40.93
Dried whey	20.00
Soybean meal, 47%	10.00
Fishmeal	10.00
Lactose	7.22
Soy protein concentrate	5.00
Poultry byproduct meal	3.22
Soybean oil	2.92
Mineral premix[1]	0.35
Vitamin premix[2]	0.20
L-Lys HCl	0.06
DL-Met	0.05
L-Thr	0.03
L-Trp	0.02
Calculated energy and nutrient levels	
ME, kcal/kg	3480
CP, %	22.53
Fat, %	6.48
Ca, %	0.80
P, %	0.73
Available P, %	0.51
Lys, %	1.50
Lactose, %	21.00

[1]Provided as milligrams per kilogram of diet: 3,000 of NaCl; 100 of Zn from zinc oxide; 90 of Fe from iron sulfate; 20 of Mn from manganese oxide; 8 of Cu from copper sulfate; 0.35 of I from calcium iodide; 0.30 of Se from sodium selenite.

[2]Provided per kilogram of diet: 2,273 μg of retinyl acetate; 17 μg of cholecalciferol; 88 mg of DL-α-tocopheryl acetate; 4 mg of menadione from menadione sodium bisulfite complex; 33 mg of niacin; 24 mg of D-Ca-pantothenate; 9 mg of riboflavin; 35 μg of vitamin B_{12}; 324 mg of choline chloride.

Table 3.2. Effect of clays on diarrhea score of pigs experimentally infected with a pathogenic *E. coli*[1]

	Treatment[2]								*P*-value					
	Sham				*E. coli*					Main effect[3]			CON vs. Clays[4]	
Item	CON	SMA	SMB	ZEO	CON	SMA	SMB	ZEO	SEM	*E. coli*	Diet	E x D	Sham	*E. coli*
d 0 to 2[5]	2.02	2.33	2.23	2.21	1.50	1.94	2.00	1.60	0.19	0.03	0.46	0.91	0.27	0.45
d 3 to 5	2.37	1.98	2.52	1.87	2.64	3.04	2.94	2.50	0.24	0.01	0.45	0.66	0.64	0.52
d 0 to 5	2.20	2.16	2.37	2.04	2.07	2.49	2.47	2.05	0.17	0.65	0.39	0.81	0.34	0.98
Pig days[6]	48	48	48	48	48	48	48	48	-	-	-	-	-	-
Diarrhea days[7]	3	4	4	4	3	8	8	5	-	-	-	-	-	-
Frequency, %[8]	6.25	8.33	8.33	8.33	6.25	16.67	16.67	10.42	-	0.13	0.14	0.08	0.64	0.13

[1]n = 8 pigs/treatment.
[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = control diet; SMA = 0.3% smectite A; SMB = 0.3% smectite B; ZEO = 0.3% zeolite.
[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E x D = interaction between *E. coli* and diet effects.
[4]Contrast between CON and all clay treatments within challenge treatments.
[5]Diarrhea score = 1, normal feces, 2, moist feces, 3, mild diarrhea, 4, severe diarrhea, 5, watery diarrhea.
[6]Pig days = number of pigs x the number of days of diarrhea scoring.
[7]Diarrhea days = number of pig days with diarrhea score ≥ 3. Statistical analysis was conducted by chi-square test.
[8]Frequency (frequency of diarrhea during the entire experimental period) = diarrhea days*100 / pig days.

Table 3.3. Effect of clays on growth performance of pigs experimentally infected with a pathogenic *E. coli*[1]

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			CON vs. SM[4]	
Item	CON	SMA	SMB	ZEO	CON	SMA	SMB	ZEO	SEM	*E. coli*	Diet	E x D	Sham	*E. coli*
d -6 to 0														
ADG, g	6.25	29.17	-2.08	-25.00	12.50	-25.00	33.33	8.33	42.4	0.80	0.86	0.38	0.87	0.84
ADFI, g	394	442	319	367	421	421	329	406	212	0.74	0.31	0.96	0.79	0.60
d 0 to 5														
ADG, g	237	180	157	187	137	132	122	85	63.71	< 0.01	0.52	0.73	0.16	0.58
ADFI, g	715	715	557	632	632	627	455	517	193	0.11	0.15	1.00	0.42	0.32
G:F[5]	0.34	0.26	0.33	0.32	0.23	0.24	0.24	0.24	0.048	0.11	0.95	0.92	0.64	0.95

[1]n =8 pigs/treatment.

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = control diet; SMA = 0.3% smectite A; SMB = 0.3% smectite B; ZEO = 0.3% zeolite.

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E x D = interaction between *E. coli* and diet effects.

[4]Contrast between CON and all clay treatments within challenge treatments.

[5]G:F was not reported for period -6 to 0 because of the negative values for ADG.

Table 3.4. Effects of clays on bacteria in lymph nodes of pigs experimentally infected with a pathogenic *E. coli*[1]

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			CON vs. Clays[4]	
Item	CON	SMA	SMB	ZEO	CON	SMA	SMB	ZEO	SEM	*E. coli*	Diet	E x D	Sham	*E. coli*
Log_{10} CFU[5]	1.05	0.74	0.65	0.60	1.87	2.12	2.03	1.69	0.30	0.01	0.88	0.90	0.44	0.87

[1]n = 64 (8 pigs/treatment).

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = control diet; SMA = 0.3% smectite A; SMB = 0.3% smectite B; ZEO = 0.3% zeolite.

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E x D = interaction between *E. coli* and diet effects.

[4]Contrast between CON and all clay treatments within challenge treatments.

[5]Log_{10} CFU/g of lymph node.

Table 3.5. Effect of clays on intestinal morphology of pigs experimentally infected with a pathogenic *E. coli*[1]

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			CON vs. Clays[4]	
Item	CON	SMA	SMB	ZEO	CON	SMA	SMB	ZEO	SEM	*E. coli*	Diet	E x D	Sham	*E. coli*
Duodenum														
VH[5]	384.6	374.6	380.6	359.1	356.6	393.8	382.5	365.0	21.06	0.99	0.78	0.80	0.64	0.40
CD[6]	264.9	276.3	263.8	262.0	273.5	257.1	259.9	275.45	39.15	0.98	0.95	0.63	0.87	0.55
VH:CD[7]	1.55	1.48	1.53	1.47	1.39	1.84	1.61	1.45	0.26	0.42	0.25	0.15	0.70	0.07
Ileum														
VH	299.0	310.7	288.7	305.8	305.4	289.8	282.1	309.9	9.15	0.64	0.36	0.72	0.85	0.45
CD	208.4	214.1	212.8	222.4	228.5	232.3	230.8	217.6	8.37	0.05	0.96	0.48	0.45	0.88
VH:CD	1.44	1.49	1.37	1.38	1.34	1.25	1.25	1.45	0.08	0.10	0.61	0.35	0.76	0.81
Colon														
CD	236.0	229.0	247. 7	227.5	228.8	244.2	223.1	216.4	73.94	0.28	0.36	0.18	0.90	0.93

[1]n = 8 pigs/treatment.
[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = control diet; SMA = 0.3% smectite A; SMB = 0.3% smectite B; ZEO =0.3% zeolite.
[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E x D = interaction between *E. coli* and diet effects.
[4]Contrast between CON and all clay treatments within challenge treatments.
[5]Villus height (μm).
[6]Crypt depth (μm).
[7]Villus height:crypt depth ratio.

Table 3.6. Effect of clays on relative amounts of sulfo- and sialomucin area of pigs experimentally infected with a pathogenic *E. coli*[1]

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			CON vs. Clays[4]	
Item	CON	SMA	SMB	ZEO	CON	SMA	SMB	ZEO	SEM	*E. coli*	Diet	E x D	Sham	*E. coli*
Ileum														
Sulfo[5]	44.31	37.59	32.86	37.95	31.28	32.31	37.38	37.10	5.80	0.52	0.97	0.73	0.37	0.65
Sialo[6]	55.69	62.41	67.14	62.05	68.62	67.69	62.62	62.90	5.80	0.52	0.97	0.73	0.37	0.65
Colon														
Sulfo	92.39	92.74	95.09	94.96	93.37	90.49	87.60	94.29	1.57	0.14	0.43	0.26	0.45	0.33
Sialo	7.61	7.26	4.91	5.03	6.63	9.51	12.40	5.71	1.57	0.14	0.43	0.26	0.45	0.33

[1]n = 8 pigs/treatment.
[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = control diet; SMA = 0.3% smectite A; SMB = 0.3% smectite B; ZEO = 0.3% zeolite.
[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E x D = interaction between *E. coli* and diet effects.
[4]Contrast between CON and all clay treatments within challenge treatments.
[5]Sulfo = % of total sulfo- and sialomucin area that is sulfamucin.
[6]Sialo = % of total sulfo- and sialomucin area that is sialomucin.

Table 3.7. Effect of clays on goblet cell number and size in ileum and colon of pigs experimentally infected with a pathogenic *E. coli*[1]

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			CON vs. SM[4]	
Item	CON	SMA	SMB	ZEO	CON	SMA	SMB	ZEO	SEM	*E. coli*	Diet	E x D	Sham	*E. coli*
Ileum														
Number[5]	25.54	23.58	23.67	25.62	27.42	25.00	32.42	26.87	3.08	< 0.01	0.16	0.06	0.49	0.71
Size (μm^2)[6,7]	29.47[b]	29.58[b]	31.88[a,b]	30.72[b]	31.00[a,b]	35.96[a]	30.65b	31.89[a,b]	0.764	0.01	0.18	0.01	0.32	0.15
Colon														
Number	28.21	24.75	27.54	25.83	27.71	28.85	26.67	24.18	12.71	0.84	0.43	0.39	0.29	0.60
Size (μm^2)	26.26[b]	27.33[a,b]	27.60[a,b]	30.37[a]	25.56[b]	28.31[a,b]	25.82[b]	27.69[a,b]	0.801	0.20	0.04	0.41	0.09	0.21

[1]n = 8 pigs/treatment.
[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = control diet; SMA = 0.3% smectite A; SMB = 0.3% smectite B; ZEO = 0.3% zeolite.
[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E x D = interaction between *E. coli* and diet effects.
[4]Contrast between CON and all clay treatments within challenge treatments.
[5]Goblet cell number; total number of goblet cells per villus, average of 3 villi.
[6]Goblet cell size, cross-sectional area.
[7]Con vs. SMA (Tukey adjustment) $P = 0.07$.

CHAPTER 4

EFFECTS OF DIETARY CLAYS ON PERFORMANCE AND BARRIER FUNCTION OF CHICKS CHALLENGED WITH *SALMONELLA ENTERICA* SEROVAR *TYPHIMURIUM*

ABSTRACT: An experiment was conducted to test for beneficial effects of dietary clays on young chicks challenged with pathogenic *Salmonella* and to explore potential mechanisms through which clays may produce benefits, with emphasis on barrier function. Two-hundred and forty, 1-d-old male commercial broiler chicks (initial BW: 41.6 ± 0.4 g; Ross x Ross line 308) were allotted in a 2 × 4 randomized complete block design with level on the battery as the block and pen as the experimental unit. Six replicates of 5 chicks/pen were assigned to each treatment. Pens were randomly assigned to 1 of 2 infection treatments (with or without *Salmonella* challenge) and four dietary treatments: basal, 0.3% smectite A (**SMA**), 0.3% smectite B and 0.3% zeolite. The *Salmonella* challenge reduced ($P < 0.05$) the growth rate of chicks fed the basal diet by 11% during d 3-7 PI. The interaction between challenge and diet occurred ($P < 0.05$) for ADFI d 7-10 and ADFI and ADG during the overall period, and the pattern was similar but not significant for other measures. Goblet cell number and size were increased ($P < 0.05$) by the *Salmonella* challenge in chicks fed the basal diet, and were reduced ($P < 0.05$) in *Salmonella* challenged chicks by feeding SMA. Villus height was reduced by the *Salmonella* challenge in the chicks fed dietary clays ($P < 0.01$) but not in chicks fed the basal diet (interaction $P < 0.05$). The concentration of interferon-γ in cecal tissues was low and the data are not reported here. Clays did not alter the concentration of α-1-acid glycoprotein in the sham-challenged group but increased it in the *Salmonella*-challenged group. In conclusion, clays restored performance of

birds challenged with *Salmonella*, SMA had effects consistent with strengthening of the mucosal barrier, and the pattern of response leads to the suggestion that different clays produce benefits through different mechanisms.

Keywords: goblet cells, chicks, clays, *Salmonella enterica* serovar *typhimurium*

INTRODUCTION

Economic loss due to enteric diseases is an important problem in the food animal industry, in spite of powerful health technologies such as all-in/all-out movements, sanitation, biosecurity, vaccines and others. Growing evidence shows that several dietary factors can help maintain health and growth performance of disease-challenged animals (Perez et al., 2011; Che et al., 2012). Also, we found that feeding a low dietary concentration of specific clays (smectite, zeolite and kaolinite) to pigs challenged with a pathogenic *E. coli* reduced diarrhea (Song et al., 2012). Clays have been used to reduce enteric disorders in humans (Carretero, 2002; Szajewska et al., 2006), but their efficacy in chicks challenged with enteric disease has not been reported. Their current use in diets for poultry and pigs is largely limited to adsorption of mycotoxins.

Several mechanisms have been proposed for the beneficial effects of clays on enteric health, including toxin binding (Phillips et al., 1988; Schell et al., 1993; Harper et al., 2010), enhancement of the immune response (Gonzales et al., 2004), strengthened barrier function (Reichardt et al., 2009; Trckova et al., 2009), mineral binding (Katsumata et al., 2003), and others (Droy-Lefaix et al., 1985; Fioramonti et al., 1987; Ward et al., 1991). All of these mechanisms are supported by controlled observations, but their relative importance under specific conditions is unknown. Potential impacts on barrier function and immune response appear especially strong and relevant to health (Gonzales et al., 2004; Trckova et al., 2009).

Our first objective is to test for beneficial effects of dietary clays on young chicks challenged with pathogenic *Salmonella*. Our second objective is to explore potential mechanisms through which clays may produce benefits, with primary emphasis on barrier function. The physical gut barrier consists of the mucus layer and the tight junctions between intestinal cells, but our focus is on the goblet cells that produce the mucus layer.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee and Institutional Biosafety Committee of the University of Illinois reviewed and approved the animal care procedures for this experiment.

Animals, Housing, and Experimental Design

Two-hundred and forty, 1-d-old male broiler chicks (initial BW: 41.6 ± 0.4 g; Ross x Ross, line 308) were allotted in a randomized complete block design with level on the battery as the block and pen as the experimental unit. Six replicates of 5 chicks were assigned to each treatment. At hatch, chicks were weighed and identified with wing bands. They were assigned to pens in a manner to equalize mean body weight across all 48 pens, and pens were assigned randomly to treatments. The chicks were housed in starter batteries with raised wire floors inside disease-containment chambers where the temperature was controlled and there was continuous lighting. The *Salmonella*-challenged chicks were in one chamber, the sham-challenged ones in a separate chamber. There were a feeder and drinker in each pen. Pens were randomly assigned to 1 of 2 infection treatments (*Salmonella* challenge versus sham challenge) and 1 of 4 dietary treatments: basal diet (**BAS**), smectite A (**SMA**), smectite B (**SMB**), and zeolite (**ZEO**) at 0.3%

inclusion in the basal diet. All the experimental diets were made from the basal and the addition of 0.3% of each dietary clay.

A primary poultry isolate of *S.enterica* serovar *typhymurium* (**ST**-10; Southern Plains Agricultural Research Center, USDA, College Station, TX), was selected. On d 0, (the day of inoculation); at 10 d of age, the inoculum was diluted to 4 x 10^8 CFU/ml using sterile phosphate-buffered saline (**PBS**). Chicks were administered a single 0.5 ml oral dose of either 2x10^8 CFU ST /dose or phosphate-buffered saline (PBS; sham) using 1-cc syringes without needles.

Ingredients, Diets, and Feeding

Clays were obtained from Milwhite, Inc. (Brownsville, TX). All diets were formulated to meet or exceed NRC (1994) recommendations and they did not include antibiotics or coccidiostatics (Table 4.1). Feed and fresh water were offered to the chicks *ad libitum*.

Data Recording and Sample Collection

Pens of chicks and feeders were weighed on d 0 (inoculation day), d 3, d 7, and d 10 post infection (**PI**) to determine ADG, ADFI and G:F. Two pens were eliminated after d 3 PI because the chicks were unable to access feed for a day because there was a defective attachment of the feeders to these pens. Due to this unwanted feed restriction, the data for performance after d 3 PI were not used. After the problem was resolved, chicks were able to access the feeders normally so the data for all the other parameters were used. On 20 d of age (d10 PI) chicks were euthanized by CO_2 inhalation, and 1 chick was randomly selected from each pen. Chicks were then dissected and ileum, cecal tissue and serum of 1 chick/pen were collected.

On d 1 through 9 PI, excreta were collected from each pen. Just before the collection, excreta pans (located under each two pens) were cleaned and fresh butcher paper was placed

under each two pens. Approximately 1 h later, all excreta from each pen were collected and weighed and stored at -20ºC until DM analysis.

The ileum was opened longitudinally and washed with PBS. Tissues were gently washed in buffered saline then fixed in Carnoy's solution for 2-3 h. Subsequently tissue samples were placed in 100% ethanol, 95% ethanol, and 70% ethanol for 30 min each and maintained in 70% ethanol until the staining process. The fixed intestinal tissues were embedded in paraffin, sectioned at 5 μm and stained with high iron diamine (**HID**) and alcian blue (**AB**), pH 2.5, as previously described (Deplancke et al., 2000). The total number of goblet cells per villus in the ileum was counted and NDP.view software was used to measure the cross-sectional area (μm^2) of individual goblet cells (goblet cell size). The measurements were performed in 7 well-oriented villi at 40x resolution. The villus height (**VH**) was measured from the tip of the villus to the valley between individual villi, and crypt depth (**CD**) measurements were taken from the valley between individual villi to the basolateral membrane and the ratio VH:CD was calculated (Figure 4.1). These measurements were made in the same villi where the goblet cell size and number were measured plus in 3 other well-oriented villi resulting in a total of 10 well-oriented villi per chick (Figure 4.2).

Cecal tissue was collected, washed with PBS and then placed in liquid N. Total RNA was isolated from tissue using the RNeasy®mini kitRNA Micro Kit (QiagenInc., Valencia, CA) according to the manufacturer's instruction for animal tissue. The quality and quantity of RNA isolates were determined using the Agilent 2100 Bioanalyzer and the ND-1000 Nanodrop spectrophotometer, respectively (Thermo Fisher Scientific). First-strand cDNA was produced from 1000 ng of total RNA per sample using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) in a total volume of 20 μL according to the

manufacturer's instruction. Then, up to 200 ul of nuclease-free water was added to each sample. Samples were stored at -20ºC until further analysis. After synthesizing cDNA, the forward and reverse primers for IFN-γ (gene of interest), and glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) (housekeeping gene) were used. The probes and primers were obtained from Applied Biosystems, Foster City, CA, which included expression assays for IFN-γ (Gg03348616_m1), and GAPDH (Gg03346982_m1). Quantitative real-time PCR was performed using TaqMAN PCR Master Mix (Applied Biosystems, Foster, CA). Reactions were run in triplicates or quadruplicates in a 384-well plate using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster, CA). Thermal cycling conditions were 50ºC for 2 min and 95ºC for 10 min, followed by 40 cycles with 15 sec at 95ºC and 1 min at 60ºC. The cycle threshold (**Ct**) values were normalized by subtracting the housekeeping gene Ct for each experimental unit (well) from the gene of interest Ct to produce the ΔCt value. The ΔCt values were analyzed statistically to test for treatment effects. A single estimate of the fold change for the housekeeping gene for each treatment was derived as follows: first the average ΔCt for the housekeeping gene for each treatment was calculated. The ΔΔCt for the gene of interest for each each bird was obtained by subtracting the average ΔCt value of basal diet in the sham-challenged group from the average ΔCt value for the housekeeping gene for each treatment. After that, the fold changes were calculated as $2^{-\Delta\Delta CT}$. The results are presented as fold change. All samples were normalized to GAPDH expression and fold change was calculated over sham-challenged controls.

The concentration of the acute phase protein α-1-acid glycoprotein (**α-1-AGP**) in the serum was measured by enzyme-linked immunosorbent assay (**ELISA**) according to the manufacturer's recommendation. Briefly, standards, control, and samples were added to the

wells coated with specific monoclonal antibody. After incubation for 45 min, the unbound substances were washed away, and an enzyme conjugated reagent was added to the wells to sandwich the acute phase protein immobilized during the first incubation. Another 45 min of incubation was followed by a wash to remove any unbound antibody-enzyme reagent, and then a substrate solution (Tetramethylbenzidine reagent) was added to the wells and incubated for 20 min for development of blue color. The color development was stopped by adding the stop solution, changing the color to yellow, and optical density was measured at 450 nm. The concentration of the acute phase protein is proportional to the optical density of the test sample. All samples were analyzed in duplicate.

Statistical Analyses

Data were analyzed by ANOVA with procedures appropriate for a randomized complete block design. Data were analyzed by the MIXED procedure (SAS Inst., Cary, NC) with blocks considered random. The statistical model included the fixed main effects of infection and diet, and their interaction. To test for the presence of outliers the Proc Univariate procedure of SAS (SAS Inst., Cary, NC) was used. Differences among the clay treatments within each challenge group were tested by pair-wise comparisons when the overall main effect of diet or the diet x challenge interaction was significant and in one case (ADFI d 3-7 PI) when $P = 0.06$.. Differences with a probability of $P \leq 0.05$ were accepted as statistically significant, whereas mean differences with *P*-values ranging from > 0.05 to 0.10 were accepted as trends.

RESULTS

Dry matter analysis of excreta was conducted but there were no effects of challenge or dietary treatments so data are not presented here.

Chick Growth and Performance

Several measures of growth performance were sharply reduced by the *Salmonella* challenge in chicks fed the basal diet, but the clays increased them to near the level of the sham-challenged group (Table 4.2; interaction ($P < 0.05$). The *Salmonella* challenge reduced ($P < 0.05$) the growth rate of chicks fed the basal diet by 11% during d 3-7 PI. The interaction between challenge and diet occurred ($P < 0.05$) for ADFI d 7-10 and ADFI and ADG during the overall period, and the pattern was similar but not significant for other measures. There were no effects of clays on G:F (Table 4.2).

Intestinal barrier:

Goblet cell number and size

The pairwise comparison indicated that *Salmonella* challenge increased both the number ($P < 0.05$; interaction) and the size ($P < 0.05$; diet) of ileal goblet cells in chicks fed the basal diet (Table 4.3). The goblet cell number was 99.50 in the chicks fed basal diet in the sham-challenged group, and 131.98 in the chicks fed basal diet in the *Salmonella*-challenged group. The goblet cell size was 23.3 μm^2 in the chicks fed basal diet in the sham-challenged group, and 29.7 μm^2 in the chicks fed basal diet in the *Salmonella*-challenged group. Feeding one of the clays (SMA) to the *Salmonella*-challenged chicks reduced ($P < 0.05$) both the number and size of those cells compared to challenged chicks fed the basal diet. The goblet cell number was 131.98

in the chicks fed basal diet in the *Salmonella*-challenged group, and 99.78 in the chicks fed SMA diet in the *Salmonella*-challenged group. The goblet cell size was 29.7 μm^2 in the chicks fed basal diet in the *Salmonella*-challenged group, and 23.4 μm^2 in the chicks fed SMA diet in the *Salmonella*-challenged group.

The SMB and ZEO improved growth performance of *Salmonella*-challenged birds, but they did not have the same effect on goblet cell number and size as SMA did.

Ileal morphometry

Villus height was reduced ($P < 0.05$) by the *Salmonella* challenge in the chicks fed dietary clays (Table 4.4). The SMA increased CD in the sham-challenged birds ($P < 0.05$). The *Salmonella* challenge unexpectedly reduced CD ($P < 0.05$) and SMA, and ZEO reduced ($P < 0.05$) VH:CD in the *Salmonella*-challenged chicks compared to chicks fed basal diet (Table 4.4).

Immune system:

mRNA expression

The Ct values for IFN-γ were too high (> 33) to be meaningful, reflecting very low mRNA concentrations, so the data are not reported here.

*Acute phase protein (*α-1-AGP*)*

The *Salmonella* challenge did not change the concentrations of α-1-AGP. Clays did not alter the concentration of α-1-AGP in the sham-challenged group but did in the *Salmonella*-challenged group (Table 4.5). Specifically, ZEO increased ($P < 0.05$) α-1-AGP concentration in the *Salmonella*-challenged group compared to the basal diet.

DISCUSSION

Our results show that the clays tested herein improve growth performance of broiler chicks challenged with *Salmonella,* with no clear differences in responses among the clays. To our knowledge, this is the first demonstration that clays in poultry diets provide benefits for birds with enteric disease, although corresponding benefits have been reported for other species. Song et al. (2012) reported that, when pigs were challenged with a pathogenic *E. coli,* feeding dietary clays including smectite, zeolite, kaolinite or combinations of them at 0.3% of the diet reduced diarrhea. Children with acute gastroenteritis when treated with smectite along with re-hydration had shorter duration of diarrhea when compared to children treated without smectite (Szajewska et al., 2006). Clays' effect on growth performance is variable. Some studies have shown no effect (Shurson et al., 1984; Poulsen and Oksbjerg, 1995), while others have shown a positive effect (Papaioannou et al., 2004; Alexopoulos et al., 2007), or even a negative effect on performance (Shurson et al., 1984) when fed to pigs. The present data showed no effect of clays when chicks were not subjected to a disease challenge.

Goblet cells are specialized epithelial cells which secrete cysteine-rich proteins that become the core components of the mucus barrier, including mucin-2 (**MUC2**), resistin-like molecule-β (**RELMβ**), and trefoil factors (Specian and Oliver, 1991). The size of these cells is determined largely by the balance between the synthesis and secretion of these proteins, with MUC2 being quantitatively the most important (Kim and Ho, 2010). This finding may explain the increase in size of goblet cells in infected chicks in the present study, as well as in previous studies (Deplancke and Gaskins, 2001; Collier et al., 2008; Fasina et al., 2010). Previous authors interpreted increases in size of goblet cells in response to pathogenic or commensal bacteria as an indication of increased mucin production (Deplancke and Gaskins, 2001;Collier et al., 2008;

Fasina et al., 2010) and therefore as a protective response. The size of goblet cells increases during the acute phase of infection, but diminishes during the chronic phase as the secretion of the cell products is not matched by new synthesis (Kim and Ho, 2010). Further, RELMβ triggers secretion of mucins, depleting the cells and reducing cell size (Krimi et al., 2008).

Differentiation of stem cells in the crypts into goblet cells is increased by inflammation, via stimulation of krüppel-like factor 4, a transcription factor that is expressed in a variety of tissues including the epithelium of the intestine and plays a role in cell differentiation but inhibits cell proliferation (Evans and Liu, 2008). More precisely, the number of goblet cells increases during the acute phase of infection (Kim and Ho, 2010). Reasons for the reduction in goblet cell number when SMA was fed in the present research are less clear, but perhaps the protective effects of the clay reduce the infectious challenge, which reduces inflammation, which reduces differentiation into goblet cells.

The present results showing that SMA reduced goblet cell size appear counter to our previous observations in pigs showing a subtle effect in the opposite direction (Chapter 3). We suggest the difference may relate to the timing of measurements. Measurements in the pig study were made 5 or 6 d after challenge with a pathogenic *E. coli*, during the acute phase of the infection, and may reflect a stronger protective mechanism in the form of greater mucin synthesis. The present measurements in chicks were made 10 d after challenge, during the chronic phase of infection, and may reflect a greater effect on secretion than on synthesis at that stage.

Overall, the present data on goblet cell number and size support the notion that one dietary clay (SMA) has effects consistent with strengthening the intestinal mucus barrier, but do not show that the other clays tested (SMB and ZEO) have that effect. The interaction was

significant for ADG and ADFI for the overall period ($P < 0.05$) where the *Salmonella*-challenged reduced ADG and ADFI but all clays provided beneficial effect by restoring these parameters in the *Salmonella*-challenged group. The pattern of response leads to the suggestion, which requires further testing, that different clays produce benefits through different mechanisms, even among the smectites.

The VH, CD, and VH:CD are used as indicators of intestinal epithelial integrity. An overall reduction in VH after *Salmonella* challenge indicates damage to the barrier and reduction in nutrient absorption that are consistent with the reduction in performance and increase in goblet cell size and number discussed earlier. However, a deepening of the crypt is also expected during infection and we did not observe it in this experiment. We also observed an unexpected reduction in VH:CD when SMA was fed in the *Salmonella*-challenged group. The values observed for VH, CD and VH:CD ratio are in the range reported by other authors (Tsirtsikos et al., 2012; Yitbarek et al., 2012; Zhang et al., 2012).

The timing of the various components of the immune response after infection of chicks with the *Salmonella* used in this experiment is not well described, and may explain the absence of effects of the challenge on measures of immunity. Interferon gamma (**IFN-γ**) is a pro-inflammatory cytokine that is induced by direct epithelial damage (Marchiando et al., 2010) and is released from macrophages during a *Salmonella* infection (Trebichavský, 1999). In this experiment we did not observe measureable expression of the IFN-γ gene, as indicated by mRNA concentrations, regardless of the infection or dietary clays (data not shown). This is in agreement with Cheeseman et al., (2008) where chicks infected with *Salmonella enteriditis* did not have an increase in mRNA expression of IFN-γ in cecal tissue at 7 or 8 d PI. However, Faber et al. (2012) showed an increase in IFN-γ mRNA expression in cecal tonsils of birds challenged

with *Salmonella* at 10d PI. Our measurements were in cecal tissue rather than cecal tonsils, which may explain the differences in results compared to Faber et al. (2012).

The lack of response of the acute phase protein to the challenge agrees with the lack of response of IFN-γ mRNA expression to the challenge at 10 d PI. Holt and Gast (2002) reported an increase in α-1-AGP 3 at 9 d after hens were challenged with *Salmonella enteriditis*; however, the timing of the immune response may differ between the mature hens in that experiment and the young chicks in the present experiment. Young chicks produce α-1-AGP after LPS injection (Takahashi et al., 1994), but that does not provide insight into the time of response after *Salmonella* infection.

CONCLUSION

In conclusion, challenging chicks with *Salmonella* reduced performance but feeding a low dietary concentration of any of 3 clays restored performance of the *Salmonella*-challenged chicks to near the level of sham-challenged chicks. This response confirms that small amounts of these clays provide benefits to chicks challenged with enteric disease, as occurs in other species. One of the clays (SMA) reduced goblet cell size and number, suggesting its benefit may be mediated through strengthening the mucus barrier. There was no indication of similar effects of the other clays, even though all clays provided beneficial effects by restoring the performance of the *Salmonella*-challenged birds, so they may function through different mechanisms.

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TABLES

Table 4.1. Ingredient composition of experimental basal diet (as-fed basis)

Ingredient	%
Corn, ground	52.85
Dehulled SBM, 47% CP	37.50
Pork Meal, 50% CP	2.00
Dicalcium Phosphate	1.50
Limestone	1.10
Salt	0.40
Poultry Trace-Mineral Premix[1]	0.15
DL-Methionine	0.20
Choline Chloride, 60%	0.10
Poultry Vitamin Premix[2]	0.20
Soybean oil	4.00

[1]Provided the following per kg of diet: Mn, 75mh (MnO); Fe, 75mg ($FeSO_4 \bullet H_2O$); Zn, 75mg (ZnO); Cu, 5mg $CuSO_4 \bullet H_2O$); I, 0.75mg (ehtylene diamine dihydroiodide); Se, 0.1mg (Na_2SeO_3).

[2]Provided the following per kg of diet: retinyl acetate, 1,514 μg; cholecalciferol, 25μg; DL-α-tocopheryl acetate, 11 mg; niacin, 22 mg; D-Ca-pantothenate, 10 mg; riboflavin, 4.4 mg; vitamin B12, 11 μg; MSBC, 2.33 mg.

Table 4.2. Effect of clays on growth performance of broiler chicks experimentally infected with *S. enterica* serovar Typhimurium[1]

	Treatment[2]									*P*-value[3]		
	Sham				SALM					Main Effect		
Item	BAS	SMA	SMB	ZEO	BAS	SMA	SMB	ZEO	s	SALM	Diet	S*D
Before Challenge												
ADFI,g	19.3	19.0	18.9	18.8	18.7	20.4	19.4	19.1	1.41	0.32	0.56	0.39
ADG,g	14.8	14.9	13.7	14.0	14.3	15. 9	15.1	14.4	1.54	0.21	0.24	0.43
G:F	0.77	0.78	0.73	0.74	0.76	0.78	0.78	0.76	0.044	0.25	0.25	0.43
d0-3												
ADFI,g	48.1	48.0	49.3	48.6	45.4	51.1	46.1	46.5	3.41	0.21	0.22	0.10
ADG,g	43.7	44.0	44.7	43.3	39.4	43.3	41.4	43.2	3.14	0.03	0.39	0.32
G:F	0.91	0.92	0.91	0.89	0.87	0.86	0.90	0.93	0.051	0.28	0.67	0.12
d3-7												
ADFI,g	64.2^{a,b}	66.1^{a}	66.1^{a}	64.9^{a,b}	58.8^{b}	64.0^{a,b}	62.6^{a,b}	65.5^{a,b}	3.56	0.02	0.06	0.23
ADG,g	54.8	55.8	55.8	54.5	48.8	52.6	52.7	54.4	2.91	0.01	0.13	0.14
G:F	0.85	0.85	0.84	0.84	0.83	0.82	0.84	0.83	0.021	0.02	0.62	0.55
d7-10												
ADFI, g	84.6^{a,b}	81.6^{a,b}	87.0^{a}	82.3^{a,b}	71.4^{b}	85.4^{a,b}	84.4^{a,b}	87.0^{a}	7.49	0.41	0.09	0.03
ADG, g	62.4	57.4	66.0	59.9	56.6	65.5	65.0	66.7	9.26	0.45	0.44	0.22
G:F	0.74	0.70	0.76	0.73	0.78	0.77	0.77	0.77	0.095	0.14	0.85	0.92
Overall, after challenge												
ADFI, g	65.5^{a}	65.3^{a}	67.3^{a}	65.2^{a}	57.1^{b}	66.6^{a}	64.2^{a,b}	66.2^{a}	3.94	0.06	0.02	0.02
ADG, g	53.7^{a,b}	52.7^{a,b}	55.5^{a}	52.8^{a,b}	47.1^{b}	53.7^{a,b}	53.2^{a,b}	54.7^{a}	3.89	0.18	0.10	0.05
G:F	0.82	0.81	0.83	0.81	0.83	0.81	0.83	0.83	0.036	0.57	0.58	0.94

[a-b]Values within a row lacking a common superscript letter are different ($P < 0.05$).

[1]n = 6 pens/trt except n=5 pens/trt for BAS and SMB in challenged group for all periods except before challenge and 0-3 PI. Initial BW: 58.24 ± 1.8 g.

[2]Sham = unchallenged; SALM = *Salmonella challenge*; BAS = basal diet; SMA = 0.3% smectite A; SMB = 0.3% smectite B; ZEO = 0.3% zeolite.

[3] SALM = *Salmonella* challenge effect; Diet = diet effect; S*D = interaction between ST and diet effects.

Table 4.3. Effect of clays on goblet cell number and size in ileum of chicks experimentally infected with *S. enterica* serovar Typhimurium[1]

	Treatment[2]									*P*-value[3]		
	Sham				SALM					Main effect		
Item	BAS	SMA	SMB	ZEO	BAS	SMA	SMB	ZEO	SEM	SALM	Diet	S*D
Ileum												
Number[4]	99.5[b]	107.5[b]	113.3[a,b]	121.4 [a]	132.0[a]	99.8[b]	116.9[a]	118.2[a]	5.27	0.26	0.16	0.04
Size (μm^2)[5]	23.3[b]	22.2[b]	28.0[a,b]	27.1[a,b]	29.7[a]	23.4[b]	28.6[a]	30.1[a]	1.52	0.07	0.04	0.56

[a-b]Values within a row lacking a common superscript letter are different ($P < 0.05$).

[1]n = 6 chicks/treatment.

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; BAS = basal diet; SMA = 0.3% smectite A; SMB = 0.3% smectite B; ZEO = 0.3% zeolite.

[3] SALM = Salmonella challenge effect; Diet = diet effect; S*D = interaction between *Salmonella* and diet effects.

[4]Goblet cell number; total number of goblet cells per villus, average of 7 villi.

[5]Goblet cell size, cross-sectional area.

Table 4.4. Effects of clays on villus height and crypt depth of chicks experimentally infected with *S. enterica* serovar Typhimurium[1]

	Treatment[2]									*P*-value[3]		
	Sham				*SALM*					Main effect		
Item	**BAS**	**SMA**	**SMB**	**ZEO**	**BAS**	**SMA**	**SMB**	**ZEO**	**SEM**	***SALM***	**Diet**	**S X D**
VH	390.85	398.65	414.73	425.98	409.37	353.08	384.18	355.13	15.67	0.05	0.67	0.24
CD	143.91^{b}	169.29	156.50	160.77	145.26	151.01	147.46	147.17	4.388	0.03	0.11	0.44
VH:CD	2.75^{a,b}	2.39^{b}	2.76^{a,b}	2.79^{a,b}	2.93^{a}	2.40^{b}	2.68^{a,b}	2.50^{b}	0.099	0.68	0.02	0.41

[1]n = 6 chicks/treatment.

[2]Sham = unchallenged; *SALM* = *Salmonella* challenged; BAS = basal diet; SMA = 0.3% smectite A; SMB = 0.3% smectite B; ZEO = 0.3% zeolite.

[3]*SALM*= *Salmonella* challenge effect; Diet = diet effect; S x D = interaction between *SALM* and diet effects.

Table 4. 5. Effects of clays on serum α-1-acid glycoprotein (α-1-AGP) levels of chicks experimentally infected with *S. enterica* serovar Typhimurium[1]

	Treatment[2]									***P*-value**[3]		
	Sham				***SALM***					**Main effect**		
Item	**BAS**	**SMA**	**SMB**	**ZEO**	**BAS**	**SMA**	**SMB**	**ZEO**	**SEM**	***SALM***	**Diet**	**S X D**
α-1-AGP (µg/mL)	182.1[b]	265.9[a,b]	151.2[b]	209.9[a, b]	187.8[b]	241.0 [a,b]	193.8 [b]	333.0[a]	21.55	0.08	< 0.01	0.08

[a-b]Values within a row lacking a common superscript letter are different ($P < 0.05$).

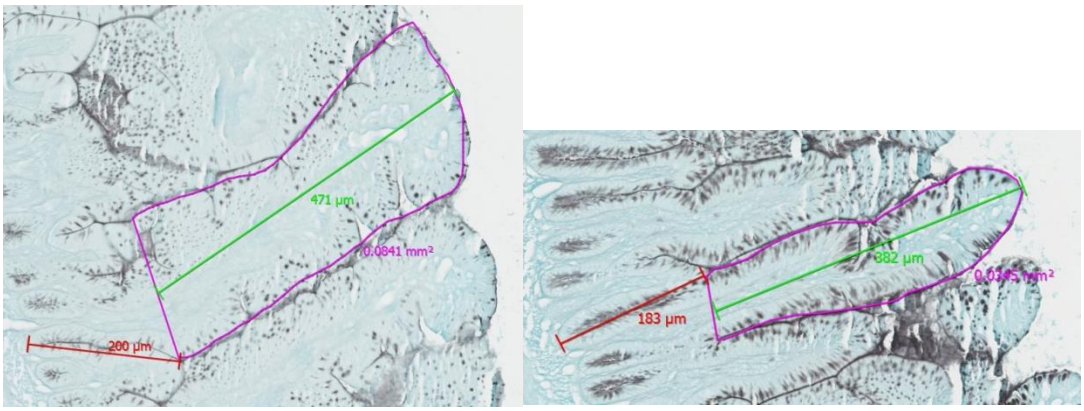
[1]n = 6 chicks/treatment.

[2]Sham = unchallenged; *SALM* = *Salmonella* challenged; BAS = basal diet; SMA = 0.3% smectite A; SMB = 0.3% smectite B; ZEO = 0.3% zeolite.

[3]*SALM*= *Salmonella* challenge effect; Diet = diet effect; S x D = interaction between *SALM* and diet effects.

FIGURES

(A) **(B)**



(C) **(D)**

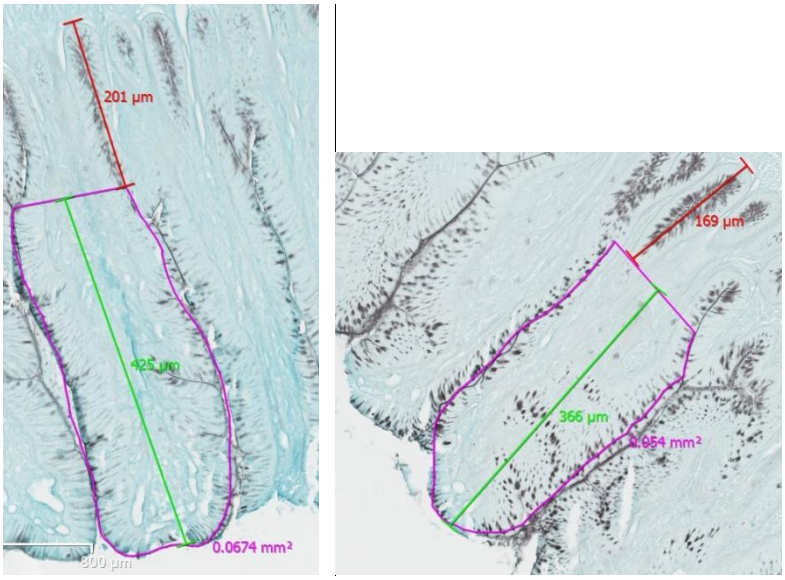
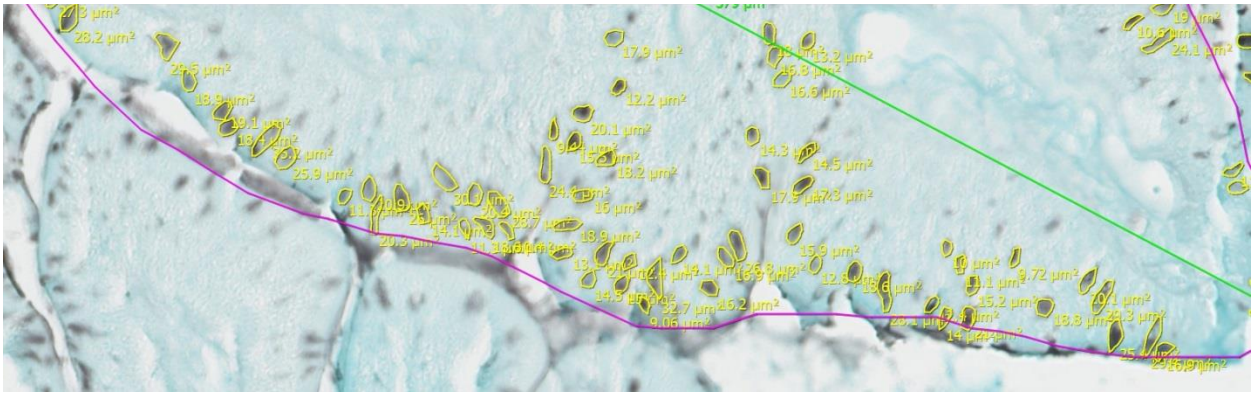
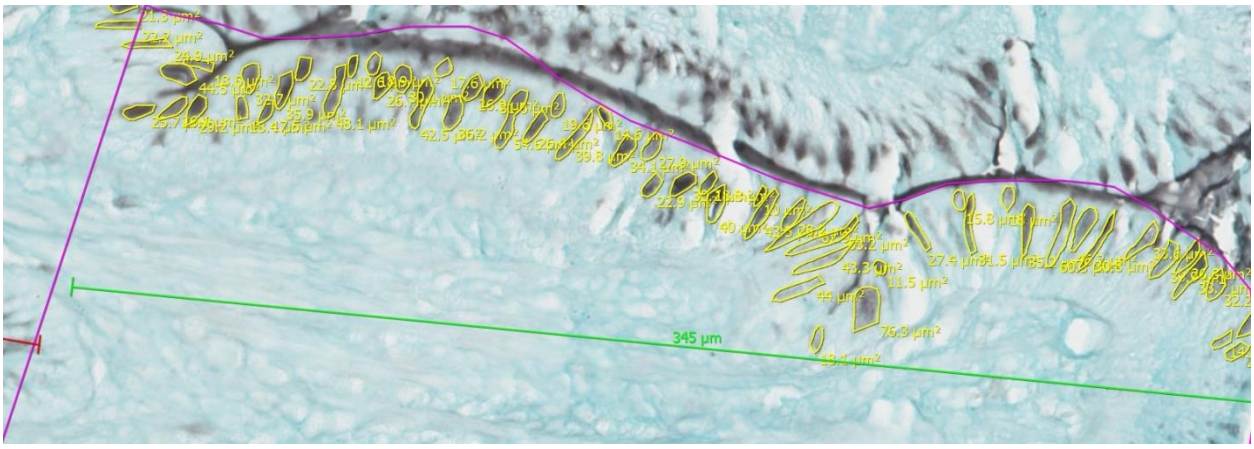


Figure 4.1. High iron diamine/alcian blue staining in ileum. The slides were scanned at 40× magnification. The measurements in green represent villus height and the measurements in red represent crypt depth. The measurements in pink are villi area (these measurements are not reported). (A) Villus of chicks from the sham-challenged group fed the basal diet. (B) Villus of chicks from the *Salmonella*-challenged group fed the basal diet. (C) Villus of chicks from the sham-challenged group fed the smectite A diet. (D) Villus of chicks from the *Salmonella*-challenged group fed the smectite A diet.

(A)



(B)



(C)

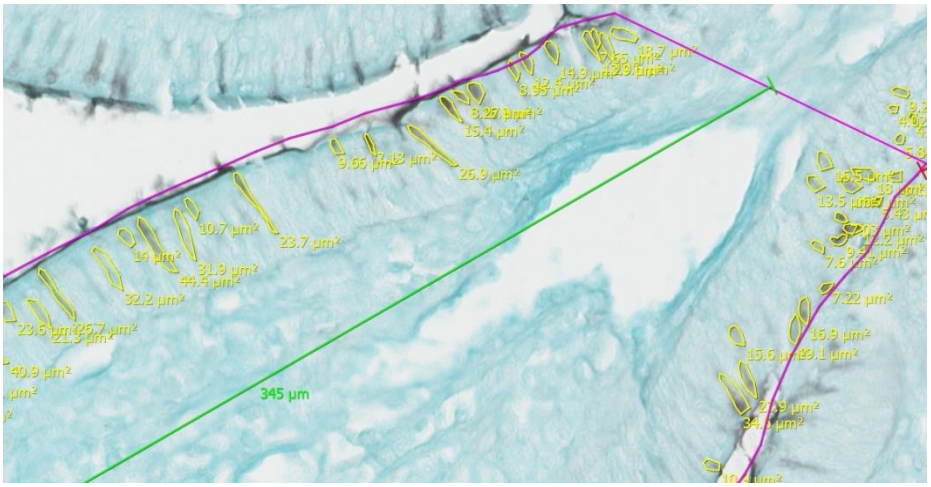


Figure 4.2.

Figure 4.2. (Cont.)

(D)

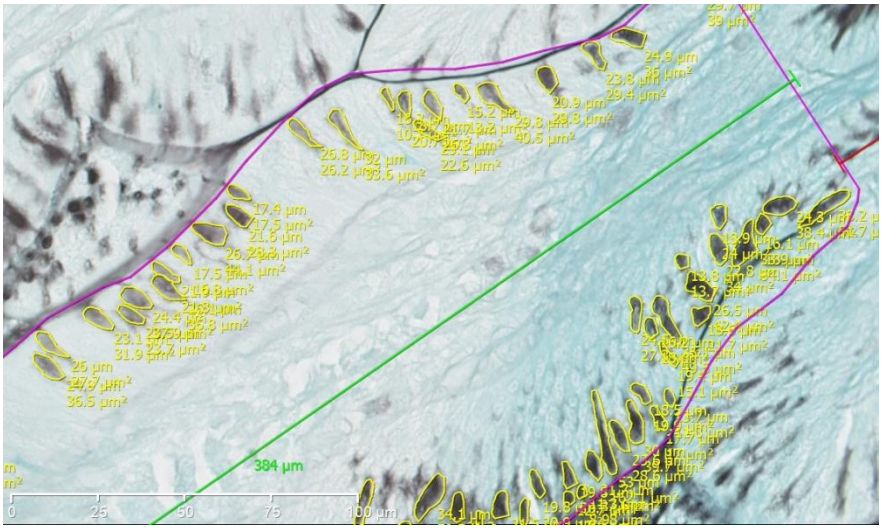


Figure 4.2. High iron diamine/alcian blue staining in ileum. The slides were scanned at 40× magnification.The measurements in yellow represent goblet cell size. (A) Villus of chicks from the sham-challenged group fed the basal diet. (B) Villus of chicks from the *Salmonella*-challenged group fed the basal diet. (C) Villus of chicks from the sham-challenged group fed the smectite A diet. (D) Villus of chicks from the *Salmonella*-challenged group fed the smectite A diet.

CHAPTER 5

EFFECTS OF SMECTITE ON MUCIN 2 (*MUC2*), TREFOIL FACTOR 3 (*TFF3*) AND RESISTIN LIKE MOLECULE BETA (*RELMβ*) GENE EXPRESSION IN LS174T-HUMAN ADENOCARCINOMA CELLS

ABSTRACT: The mechanisms through which dietary clays alleviate diarrhea is unknown; however, there is strong indication in the literature that it could be by improving barrier function. In order to explore the potential mechanisms through which smectite A (**SMA**) may produce the beneficial effects previously observed *in vivo,* an *in vitro* project was conducted. Cell cultures were performed using a human colorectal adenocarcinoma cell line (**LS174T**), which has a goblet cell-like phenotype. Three different times LS174T cells were cultured in triplicate (each time) in the absence or presence of 3 concentrations (0.05, 0.10, and 0.50%) of SMA and incubated for 24h. Expression of genes encoding goblet cell secretory products mucin 2 (***MUC2***), resistin-like molecule β (***RELMß***), and trefoil factor 3 (***TFF3***) were determined by quantitative reverse transcriptase-polymerase chain reaction (**qRT PCR**). The data were analyzed individually (i.e. each cell culture separately) as well as combined (i.e. the data of all 3 cell cultures). For the overall data, the expression of *RELMB* by LS174T cells was increased ($P < 0.05$) in the presence of 0.10% SMA to 1.44 fold change relative to the control. The expression of *MUC2* was reduced ($P < 0.05$) by fold change 0.77 in 0.10% SMA. Because the magnitude of response to SMA by LS174T cells was greater for the expression of *RELMβ* (41% compared to 25% for *MUC2* expression) as well as it was consistent with the goblet cell size data (Chapter 4), then we focused the further measurements on RELMß. Another cell culture was performed in triplicate in LS174 T cells and after 96 h of incubation without or with SMA (0.05% and 0.10%)

the medium and cell lysate were collected and analyzed using Western Blot. The media was replaced after 48 h of incubation. The SMA reduced the amount of the protein in the cell lysate samples but increased it in the medium. This result indicates that the clay increased secretion of RELMß, concentrating this protein in the medium and depleting the cell. Together, the data show that SMA stimulates more mRNA production and secretion of RELMβ, consistent with an improvement in intestinal barrier as a potential mode of action of SMA for reduction of diarrhea *in vivo*.

Keywords: LS174T cell, RELMβ, smectite.

INTRODUCTION

Enteric disease is a problem in the animal industry and leads to economic loss. Several dietary factors, including clays, can help maintain health and growth performance of challenged animals (Song et al., 2012) as well as hasten recovery of humans facing enteric disorders (Carretero, 2002; Szajewska et al., 2006). Several mechanisms have been proposed for the beneficial effects of clays on enteric health, including toxin binding (Phillips et al., 1988; Schell et al., 1993; Harper et al., 2010), antimicrobial effects (Xia et al., 2004; Tong et al., 2005), and strengthening of the intestinal barrier (Gonzales et al., 2004; Xia et al., 2005; Reichardt et al., 2009), but the importance of each of them is unknown. Potential impacts on barrier function appear especially strong and relevant to health (Droy-Lefaix et al., 1985; Droy-Lefaix, 1987; Gonzales et al., 2004; Trckova et al., 2009). The physical gut barrier consists of the mucus layer and the tight junctions between intestinal cells, but our focus is on the goblet cells that produce the mucus layer.

Goblet cells are specialized epithelial cells that secrete cysteine-rich products (Moncada and Chadee, 2002) such as mucin 2 (**MUC2**), trefoil factor 3 (**TTF3**) and resistin like molecule

beta (**RELMβ)**. These products are involved in maintaining integrity of the gastrointestinal mucosal surface. Goblet cells have been reported to be more abundant in the colon and this may indicate the importance of secreted mucus in that region (Specian and Oliver, 1991).

The genes of the *MUC* family encode the peptide backbones of mucins. There are secreted (*MUC2*, *MUC5A*, *MUC5B*, *MUC6*, and *MUC7*) and membrane bound (*MUC3*, *MUC4*) mucins (Corfield et al., 2000).The MUC2 is the major secreted mucin in the small and large intestine. It forms trimers that are fundamental to the complex network that is formed along the epithelial cell lining in the small and large intestine, and is central to the protective property of the mucus (Kim and Ho, 2010).

The TFF are secreted in the gastrointestinal tract (Thim et al., 2002) and usually associated with the mucin layer where they have a healing function (Thim, 1997). There are 3 trefoil factors: TFF1, TFF2, and TFF3. The *TFF1* is expressed mainly in the stomach, *TFF2* mainly in the stomach, duodenum, and pancreas, and *TFF3* mainly in the intestine (Thim, 1997). The *TFF3* is almost exclusively expressed by goblet cells. The *TFF* co-localizes with *MUC2* (Taupin and Podolsky, 2003) and is involved in repair of mucosal epithelial damage in order to maintain barrier function and prevent inflammation (Kim and Ho, 2010). The TFF is the second most abundant goblet cell product; it increases viscosity and contributes to the stability of mucin.

The RELMβ is another product of goblet cells in the intestine and its concentration is greater in the large intestine (Kim and Ho, 2010). The expression of *RELMβ* is induced upon bacterial colonization; thus, it is associated with mucosal injury (McVay et al., 2006) in certain pathological conditions. The RELMβ likely plays a defensive role against nematode intestinal infection (McVay et al., 2006) and also triggers protective and pro-inflammatory mechanisms in animals exposed to chemically induced colitis (Kim and Ho, 2010).

Our objective was to determine if clays, specifically smectite A (**SMA**) have an impact on intestinal barrier function. We tested if SMA alters gene expression of any of these genes: *MUC*2, *TFF3* and *RELMβ*. Protein synthesis is the ultimate outcome of expression of genes that encode that protein segment so we also tested the protein production by *RELMβ.*

MATERIALS AND METHODS

Treatments

One kind of smectite (designated SMA in Chapter 4) was provided by Milwhite, Inc. (Brownsville, TX) for use in this research. The clay treatment was prepared at 3 different concentrations (0.05, 0.10, and 0.50%) for the gene expression data. The photos of the cell culture with the 0.50% concentration showed a considerable layer of clay covering the cells, leading to concern that the cells may not function normally. For that reason, the data from that treatment are not presented and that treatment was eliminated from the measurement of the gene product. The lower concentrations of clay also partially covered the cells but in a more modest way. The two lower clay concentrations appear to be in the range that might be encountered *in vivo*. The SMA was autoclaved prior to use to avoid contamination and Minimum Essential Medium (**MEM**) was added to achieve the desired concentration.

Cell Culture

The human colorectal adenocarcinoma cell line (**LS174T**) passage 114, was obtained from the American Type Culture Collection (**ATCC**). The cells were maintained in the culture 75 ml flasks with MEM including 100 IU penicillin/mL and 100 µg streptomycin/mL and 10% fetal bovine serum (FBS, HyClone Laboratories, Inc., Logan, UT) at 37°C in 5% CO_2. The cells were subcultured (i.e., transferred to a fresh culture medium) in the flasks until the total number

of cells was sufficient for the experiment. After getting 80% confluence, the cells were washed with Hank's Buffered Salt Solution (**HBSS**) and briefly rinsed with 2-3 ml of 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to release the cells from the bottom of the flasks. The collected cells were suspended in fresh MEM and counted using a hemocytometer (Fisher Scientific Inc., Pittsburgh, PA). The cells were suspended in the MEM, subcultured in 12 or 24-well plates at a density of 4×10^6 cells/well for 12-well plate, and 2×10^6 cells/well for 24-well plate and incubated for 24h at 37^{o}C in a humidified 5% CO_2 incubator to allow cells to adhere to the plate. The non-adherent cells were washed away with warm HBSS. Then adhered cells were incubated with fresh medium containing different treatments as described above. The control treatment was MEM media only. All treatments were conducted with 3 replicate wells. Each well was an experimental unit. Cells were harvested after 24 h incubation for gene expression measurements and after 96 h incubation for protein measurements and stored at -80 ºC for RNA isolation. The media was harvested after 96 h incubation for protein measurements but the media was changed after 48 h incubation so the media that was collected reflects only the last 48 h of incubation.

Quantitative real -time reverse transcription polymerase chain reaction (qRT-PCR)

The ß-glucuronidase (**GUSß**) catalyzes breakdown of complex carbohydrates and was used as a housekeeping gene. All data obtained from the target genes were normalized according to the expression of the housekeeping gene.

Total RNA was isolated from cells using the RNeasy®mini kitRNA Micro Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instruction for animal cells. The quality and quantity of RNA isolates were determined using the Agilent 2100 Bioanalyzer and the ND-1000 Nanodrop spectrophotometer, respectively (Thermo Fisher Scientific). First-strand cDNA was

produced from 1000 ng of total RNA per sample using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) in a total volume of 20 µL according to the manufacturer's instruction. Then, up to 200ul of nuclease free water was added to each sample. Samples were stored at -20ºC until further analysis. The probes and primers were obtained from Applied Biosystems, Foster City, CA, which included expression assays for *MUC2* (Hs00159374_m1), *TFF3* (Hs00173625_m1), *RELMß* (Hs00395669_m1), and *GUSß* (Hs99999908_m1). Quantitative real-time PCR was performed using TaqMAN PCR Master Mix (Applied Biosystems, Foster, CA). Reactions were run in triplicates or quadruplicates in a 384-well plate using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster, CA). Thermal cycling conditions were 50ºC for 2 min and 95ºC for 10 min, followed by 40 cycles with 15 sec at 95ºC and 1 min at 60ºC. The cycle threshold (**Ct**) values were normalized by subtracting the housekeeping gene Ct for each experimental unit (well) from the gene of interest Ct to produce the ΔCt value. A single estimate of the fold change for each treatment was derived as follows: first the average ΔCt for each treatment was calculated. The ΔΔCt for each treatment was obtained by subtracting the average ΔCt value of control from the average ΔCt value of each clay treatment. After that, the fold changes were calculated as $2^{-\Delta\Delta CT}$.

Immunoblot analysis

For the cell lysate analysis, at the end of the incubation period (96h), cells were washed with HBSS then lysed with 400 µl 1X Cell Lysis Buffer (Cell Signaling Technology, Inc., Danvers, MA) with protease inhibitor cocktail (Sigma Aldrich Co., St. Louis, MO). Following incubation for 5 min on ice, cells were collected by scraping, and then sonicated. The extract was centrifuged for 10 min at 14,000 x g in a cold microfuge and the supernatant was removed for use. An aliquot containing 40 µg protein was mixed with the same volume of sample solubilizing

buffer (**SSB**). To prepare 1ml of SSB 50 µl of β-mercaptoethanol were added to 950 µl of 2×laemmli sample buffer (Bio-Rad Laboratories, Inc., Hecules, CA), then heated for 5 min at 95°C, cooled, and electrophoresed on 10-15% polyacrylamide gel at 100v for 1 h (SDS-PAGE; Bio-Rad, Hercules, CA). The proteins in the gel were electrophoretically transferred to a polyvinylidene fluoride (**PVDF**) membrane (Fisher Scientific Inc., Pittsburgh, PA) at 100 volts for 1h. Then the protein-binding sites on the membrane were blocked with skim milk reconstituted in Tris-buffered saline (8-10% final concentration) with 0.1% Tween 20 (**PBST**) for 1 h at room temperature . The bound proteins were probed with the primary antibodies, RELMβ (Rabbit anti-human at a dilution 0.2 µg/ml) and β-actin (mouse monoclonal antibody at a dilution of 1:800) overnight at 4°C. Following incubation, the blots were extensively washed PBST and incubated with respective (goat anti-rabbit and rabbit anti-mouse) horseradish peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:2000 for 1 h at room temperature. After thorough washing in PBST, blots were developed using the Super Signal West Femto System (Thermo Scientific, Rockford, IL) 1:1 and incubated for 3 min in the dark. Membranes were photographed using Image Quanto LAS 4000 (GE, Uppsala, Sweden) at -25ºC and the densitometry of the bands was performed using Image J analysis software (NIH).

For the media analysis, the procedure was as described above except that, at the end of the incubation period (96h), cells were washed with HBSS and the medium was collected and lyophilized, then re-suspended in 500 µl of PBS 1× solution, then quantified for total protein before electrophoresis.

Statistical Analysis

Data were analyzed using the GLM procedure (SAS Institute Inc., Cary, NC). The model included treatments only, and each level of clay was compared to control. Each well was considered an experimental unit. An alpha value of 0.05 was used to assess significance among means.

RESULTS

The mRNA expression of *RELMβ* was numerically higher by at least 38% on one or the other of the concentrations of clay in all 3 of the cultures (Table 5.1), reaching significance ($P \leq 0.05$) in 2 of them and in the overall data ($P < 0.01$). The expression of *MUC2* was reduced ($P = 0.02$ overall) by a smaller margin in the presence of 0.10% clay. These data provide evidence that the clay did not alter the expression of *TFF3*.

The relative abundance of the gene product, RELMβ, in control and clay treated cells was assessed by Western Blot analysis. When LS174T cells were grown in the presence of 0.05% SMA for 96h there was an increase of RELMβ secretion into the medium (Figure 5.1). A predominant band was detected in cell lysate of LS174T cells exposed to MEM only (control treatment) for 96h, but not in the cell lysate exposed to clay, thus confirming that the clay (in this case, at both concentrations: 0.05 and 0.10%) depleted the cells of RELMβ. Confirming data are shown (Figure 5.1).

DISCUSSION

Clays have been used mostly in human practice to ameliorate diarrhea but they are also used in the pig and poultry industry with some success. Song et al. (2012) fed smectite, zeolite

and kaolinite at 0.3% of the diet to pigs challenged with a pathogenic *E.coli* and observed a reduction in diarrhea in those pigs. The mode of action of clays in reducing diarrhea is not well understood, even though possible modes of action have been proposed such as: toxin binding (Phillips et al., 1988; Harper et al., 2010), antimicrobial effects (Xia et al., 2004; Tong et al., 2005), strengthening of the intestinal barrier (Gonzales et al., 2004; Xia et al., 2005; and Reichardt et al., 2009).

However, there are strong indications that clays may strengthen the intestinal barrier. Smectites have beneficial effects on gastrointestinal health of animals (Fioramonti et al., 1987; Gonzales et al., 2004; Song et al., 2012) and humans (Szajewska et al., 2006) either in antidiarrheal effect, improved of colonic histology, or reduced duration of diarrhea.

One of *E.coli*'s virulence factors is binding the enterocyte via fimbriae. If clays increase mucin production that makes it less likely that *E.coli* will reach the enterocyte, thus preventing or alleviating diarrhea.

This project was conducted in order to determine if SMA alters the intestinal barrier by increasing expression of any of 3 genes involved in maintaining that barrier. When LS174T cells were exposed to SMA at 0.10%, there was an increase in *RELMβ* and a decrease in *MUC2* mRNA expression. The magnitude of the response was greater for the up-regulation of *RELMβ* (41%) than for the down-regulation of *MUC2* (25%) so we focused measurement of gene products on RELMβ only. Moreover, the up-regulation of *RELMβ* is consistent with our previous results (Chapter 4) with goblet cell size data. Because RELMβ is involved in maintenance of colonic epithelial cell barrier function we can conclude that SMA has a protective effect in maintaining the mucosal defense barrier by up-regulating *RELMB.*

The RELMβ is a secreted protein and the protein measured in the Western Blot was collected from the cell lysate (96 h incubation) as well as media (48 h incubation). The cells treated with SMA secreted more RELMβ so there was more RELMβ in media collected in the last 48 h of incubation and less in the cell lysate. These results of increased signal (mRNA expression) and increased secretion of RELMβ demonstrate that SMA has a protective effect on intestinal barrier.

The relationship of the apparent down-regulation of *MUC2* to other observations, including reduced diarrhea (Song et al., 2012) is unclear. Diosmectite 0.1% has been shown to up-regulate *MUC2* levels in HT29-MTX cells (Gonzales et al., 2004), in contrast to the current results. The simple fact that different clays are used in different experiments may explain the apparently conflicting results. It has been our experience that different clays have different effects even though they sometimes are classified in a common category, such as “smectites”.

CONCLUSIONS

When LS174T cells were exposed to SMA, the expression of *RELMβ* was up-regulated and the secretion of RELMβ was increased. Combined, these data suggest that clays help improve barrier function even in the absence of an enteric challenge *in vitro*. To the best of our knowledge this is the first time that expression of *RELMβ* was measured in LS174T cells exposed to SMA or even to clays.

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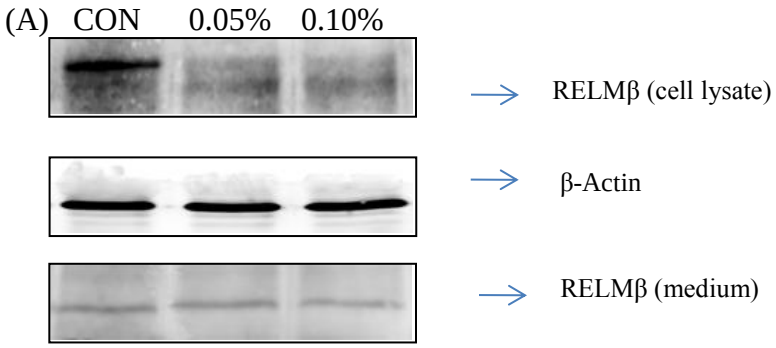
TABLE

Table 5.1. Effect of different concentrations of SMA[1] on gene expression in LS174T cells, in fold change[2]

		Clay, %				*P*-values		
Cell Cultures	Genes	0	0.05	0.10	SEM	Con[3] vs. 0.05%	Con vs. 0.1%	Con vs. SMA
1	*MUC2*[4]	1.04	0.85	0.60	0.186	0.33	0.04	0.08
1	*TFF3*[5]	1.01	1.10	1.04	0.117	0.50	0.83	0.54
1	*RELMB*[6]	1.03	1.56	1.27	0.164	0.01	0.18	0.03
2	*MUC2*	1.01	0.84	0.75	0.121	0.21	0.06	0.07
2	*TFF3*	1.19	1.07	1.15	0.131	0.36	0.76	0.49
2	*RELMB*	1.03	1.12	1.68	0.125	0.47	<0.01	<0.01
3	*MUC2*	1.01	1.10	0.91	0.171	0.60	0.58	0.99
3	*TFF3*	1.10	1.04	1.13	0.269	0.83	0.91	0.95
3	*RELMB*	1.00	1.12	1.38	0.240	0.63	0.14	0.26
All	*MUC2*	1.02	0.94	0.77	0.100	0.44	0.02	0.06
All	*TFF3*	1.10	1.07	1.11	0.110	0.33	0.90	0.64
All	*RELMB*	1.02	1.24	1.44	0.120	0.07	<0.01	<0.01

[1]SMA = smectite A.
[2]Data are means of 11 observations; the first culture was conducted in triplicates and the last 2 in quadruplicates.
[3]Con = control.
[4]MUC2 = mucin 2.
[5]TFF3 = trefoil factor 3.
[6]RELMB = resistin-like molecule beta.

FIGURES



(B)

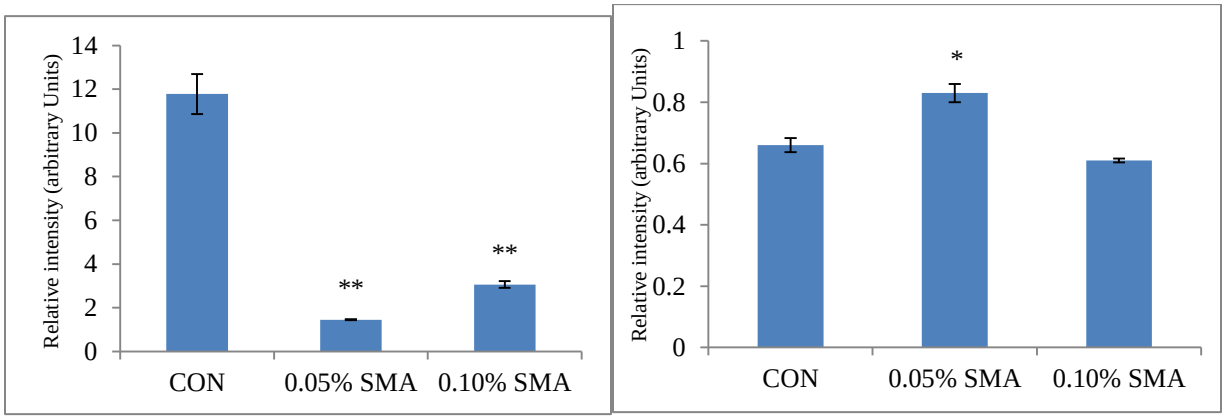


Figure 5.1. Effect of SMA at different concentrations in the production of RELMβ. A predominant band was detected in cell lysate (A) of LS174T cells exposed to MEM only (control treatment) for 96 h incubation. There was an increase in RELMβ secretion by LS174T cells when exposed to 0.05% SMA for 48 h incubation in the medium. * $P < 0.01$; ** $P < 0.001$(B).

CHAPTER 6

GENERAL CONCLUSIONS

In the livestock industry, pigs and poultry are susceptible to *E.coli* and *Salmonella* infection (among other enteric infections). These enteric diseases can damage the intestinal barrier and reduce productive performance. Dietary clays that are mainly used as mycotoxin binders are also known to reduce diarrhea. It is important to determine the mode of action of dietary clays during an enteric infection. Three approaches were taken in order to explore potential mechanisms related to intestinal barrier. The first one consisted of pigs challenged with a pathogenic *E.coli,* the second one of chicks challenged with *Salmonella*, and the third one of an *in vitro* study where LS174T cells were exposed to clay.

During an acute phase of challenge (up to 5 d PI) with *E.coli,* pigs had increased diarrhea, decreased ADG, increased crypt depth (CD), increased bacterial translocation from intestinal lumen to mesenteric lymph nodes, and increased goblet cell size and number compared to unchallenged controls. Similarly, chicks during a more chronic phase of challenge (up to 10 d PI) with *Salmonella*, had decreased growth performance, reduction in villus height (VH) and increased goblet cell size and number. Thus, these data suggest that damage to the intestinal barrier causes reduction in growth performance.

The use of dietary clays ameliorates the deleterious effects of an enteric infection. The goblet cell size and number were increased during the acute phase of infection in challenged pigs and may reflect protection that was observed in our previous research where measurements were taken throughout the course of the disease and clays reduced the frequency of diarrhea. The lack

of response to dietary clays in the most recent trial may be due to the shorter duration of this one when compared to the previous one.

Clays restored performance of challenged chicks and smectite A (**SMA**) reduced goblet cell size and number. These observations seem superficially to contradict the observations in the challenged pigs; however, the goblet cell measurements were made during acute infection in pigs and during the chronic phase (recovery) of chicks.

The 3 approaches taken to determine potential modes of action of dietary clays have proven to be successful and interrelated, according to the following interpretation. Western blot analysis showed that SMA increased the quantity of RELMβ in the medium but reduced it in the cells. We interpret that pattern of response to suggest that SMA stimulated the secretion of RELMβ and that the subsequent reduction in the concentration of the protein within the cells stimulated its synthesis, as reflected in the increased mRNA. The increased production of goblet-cell products is expected to strengthen the intestinal mucus barrier, consistent with our previous finding of reduced diarrhea in pigs and our present observation of increased growth rate in challenged chicks. The more effective barrier reduces the invasion of pathogens, thus reducing inflammation and thereby diminishing the stimulation of differentiation of stem cells to goblet cells. This pattern is consistent with our observation of fewer goblet cells when clay was fed. Goblet cell size is determined by the balance between synthesis and secretion of products. The literature indicates that during the acute phase of an infection there is increased synthesis of mucins but during the chronic phase synthesis declines while secretion continues, resulting in depletion of the cells. If clays enhance these processes, that would explain the clay effects of increased goblet cell size in pigs during the acute phase of infection and also the reduced size in chicks during the chronic phase. Overall, the results of the three approaches suggest that some

dietary clays protect against enteric disease by strengthening the mucus barrier, which occurs in turn by modifying goblet cell function. These findings do not eliminate other potential modes of action; in fact some of these data suggest that some clays work through other mechanisms.

Antibiotics have been widely used in the swine and poultry industry as growth promoters. However, the use of antibiotics as growth promoters has been banned from Europe since January 2006 due to the potential transference of antibiotic resistant genes from animals to humans. It is important to find reliable alternative strategies to maintaining pig and poultry health. These data indicate that clays are potential feed additives to be used in situations where the use of antibiotics as growth promoters is restricted.

It is necessary to use caution when extrapolating these data regarding the modes of action of clays. The present data suggest that the clays had similar clinical effects as all of the restored performance in *Salmonella*-challenged chicks but not all of them had similar modes of action so the modes of actions of clays may vary among different types of clays and even among smectites.

Future research is needed in order to specifically determine the mode of action of particular dietary clays that have beneficial effects during an enteric challenge. First of all, it is important to consider the time of sampling and duration of infection. Also, other clays should be tested for potential beneficial effects, as well as other genes. The intestinal barrier also encompasses tight junctions so genes related to tight junctions' integrity should also be considered. These genes are not restricted to but could encompass: *e-cadherin, zonula occludens, occludin,* and *claudin*. Also, the effects of clays on transepithelial resistance should be explored. During an enteric infection it is expected that the integrity of the intestinal barrier is reduced,

resulting in reduced transepithelial resistance thus an increased ion transport taking place across the epithelium.

Considering modified clays, it is important to attribute the beneficial effects either to the clay, to the modification or to both as in a synergistic mechanism. In order to do that, the experiments should be designed in a proper way including a basal diet, addition of dietary clays only, addition of the modified clays and if possible one dietary treatment should include the modification only (such as Cu, Zn etc). That would give the producer a chance to evaluate financially what approach is more economical. For instance, if the beneficial effect is due to clay alone, it could be cheaper to feed natural clay and not have to process it. The extrapolation of the data from pigs and chicks to human should be done with caution because the level of inclusion of clays is typically higher in research done with humans.

Besides these approaches, experiments in commercial farms are also necessary. Under experimental conditions, the environment is controlled and stressful situations are reduced to a minimum (such as low number of animals/pen, adequate temperature, single pathogen infection etc). In a commercial farm situation there are breakouts that often involve a combination of pathogens. It is necessary to determine the appropriate dosage of clays and duration of feeding in this case in order to see beneficial effects in ameliorating the detrimental effects of an enteric infection.

In conclusion, this research provides novel data regarding bacterial translocation in pigs and goblet cell size and number in pigs and chicks, and indicates that dietary clays can ameliorate enteric disease damage especially in the chronic phase of an infection and it does so by altering gene expression and protein synthesis, consequently goblet cell function and increasing mucus barrier.